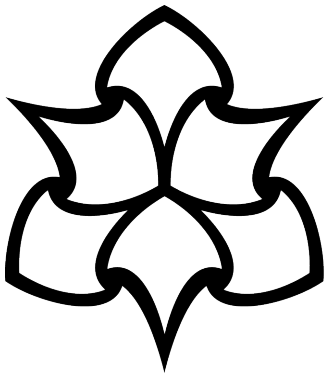

THE EFFECTS OF SMOKING AND HYPOXIA ON SKELETAL MUSCLE STRUCTURE AND FUNCTION

by

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Abstract

Chronic obstructive pulmonary disease (COPD) is increasingly common and in 80% of the patients it is caused by cigarette smoking, often over several decades. Exercise intolerance is a major problem in COPD and there is accumulating evidence that this is not solely due to reduced lung function but that it also involves skeletal muscle. It is not known if changes in skeletal muscle tissue occur before clinical symptoms of COPD are apparent. Therefore the main aim of the work described in this thesis was to gain insight into the extent to which cigarette smoking and hypoxaemia affect the functional and morphological changes of skeletal muscle.

Percutaneous electrical stimulation of the quadriceps muscle (2 minutes 30 Hz with duty cycle of 0.5; 1 s on, 1 s off) was used to assess fatigue characteristics to avoid possible problems of differences in voluntary activation.

In non-smoking participants, women were generally more fatigue-resistant than men. Young men with a relatively short smoking history were more susceptible to fatigue than non-smoking, age- and physical activity-matched peers. In a larger cohort of smokers spanning a wide range of age and smoking history, it appeared that this lower fatigue resistance was not related to smoking history. The similar contractile speed in smokers and non-smokers suggests that factors other than differences in muscle fibre composition underlie the reduced fatigue resistance in smokers. The reduced fatigue resistance might be more related to a possible acute, and possibly reversible, impairment of smoking on the oxygen supply or the ability to use oxygen for aerobic energy generation by the muscle.

Subsequently, the effects of chronic hypoxia on determinants of oxygen supply and oxidative capacity were studied. Adaptations within the rat plantaris muscle to chronic hypoxia were region- rather than fibre type-specific. Hypoxia resulted in atrophy in all regions of the muscle. However, capillary proliferation only occurred in the deep, oxidative region, and mitochondrial biogenesis was restricted to the superficial, glycolytic region. Model calculations indicate that these adaptations to hypoxia prevent the occurrence of anoxic areas in the deep region, but that in superficial regions tissue oxygenation becomes more problematical during hypoxia.

These results are discussed in relation to their possible relevance in understanding muscle function in COPD patients.

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Wüst RCI, Gilliver SF & Degens H (2008). Specific tension and the force-velocity curve are changed in response to chronic hypoxia. *Beijing Joint Conference of Physiological Sciences, Abstract book* P1-07-02. (Beijing, China).

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List of abbreviations

α_M	solubility of oxygen in skeletal muscle
a/P_o	curvature of the force velocity curve
ABS	avidin–biotin–peroxidase complex
ADP	adenosine diphosphate
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphatase
BMI	body mass index
BSA	bovine serum albumin
C/F	capillary to fibre ratio
CD	capillary density
CFD	capillary fibre density
CO	carbon monoxide
CO_2	carbon dioxide
COHb	carboxyhaemoglobin
COPD	chronic obstructive pulmonary disease
CSA	cross-sectional area
ΔA_{660}	absorbance units at 660 nm
DAB	3,3'-diaminobenzidine-tetrahydrochloride
D_{Mb}	radial diffusion coefficient of myoglobin ($0.27 \cdot 10^{-4}$ mm^2 s^{-1})
DO_2	diffusion coefficient for oxygen in skeletal muscle
EDL	extensor digitorum longus
EMG	electromyogram
FCSA	fibre cross-sectional area
FEV_1	forced expiratory volume in 1 s
FI	fatigue index
GPDH	glycerol-3-phosphate dehydrogenase
Hb	haemoglobin
HCl	hydrochloric acid
HEPES	n-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid
HIF1-α	hypoxia-inducible factor 1-alpha
HIV	human immunodeficiency virus
IL-6	interleukin-6
LCFR	local capillary to fibre ratio
$\log_R SD$	log-transformed standard deviation of the radius of the capillary domain

Mb	myoglobin
$[MbO_2]_R$	concentration of oxygenated myoglobin at the sacrolemma
MHC/MyHC	myosin heavy chain
MyLC	myosin light chain
MRC	maximal rate of contraction
MRR	maximal rate of relaxation
MTC	maximal torque capacity
MVC	maximal voluntary contraction
NAD(H)	nicotinamide adenine dinucleotide
NBT	nitro blue tetrazolium
NMJ	neuromuscular junction
PaO_2	arterial oxygen pressure
PBS	phosphate buffered saline
PCr	phosphocreatine
Pi	inorganic phosphate
PO_2	partial pressure for oxygen
PO_{2cap}	critical oxygen tension at the capillary required to prevent the development of anoxic tissue areas when the muscle is working at $V'O_{2max}$
PO_{2crit}	critical oxygen tension required homogeneously around a muscle fibre to prevent the development of anoxic cores within the fibre when it is working at $\dot{V}O_{2max}$
PGC-1α	peroxisome proliferator-activated receptor-gamma coactivator-1-alpha
PPAR-γ	peroxisome proliferator-activated receptor-gamma
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDH	succinate dehydrogenase
SERCA1	sarco/endoplasmic reticulum Ca^{2+}-ATPase expressed in type II fibres
SERCA2	sarco/endoplasmic reticulum Ca^{2+}-ATPase expressed in type I fibres
SR	sarcoplasmic reticulum
TNBT	tetranitroblue tetrazolium
TNF-α	tumour necrosis factor-alpha
TRIS	tris (hydroxymethyl)aminomethane
T-tubules	transverse tubules
VA	voluntary activation
VEGF	vascular endothelial growth factor
$\dot{V}O_{2max}$	maximal oxygen uptake per unit time

Contributions

The following people have contributed to the thesis:

Chapter 1:

Hans Degens	Contributed to the writing of page 2-7

Chapter 2:

Chris I. Morse	Contributed to the development of the experimental protocol, and revised the chapter
Arnold de Haan, David A. Jones and Hans Degens	Contributed to the design of the study, development of the experimental protocol and revised the chapter

Chapter 3:

Chris I. Morse	Contributed to the development of the experimental protocol, and revised the chapter
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Chris I. Morse	Contributed to the development of the experimental protocol, and revised the chapter
Arnold de Haan, Jörn Rittweger, David A. Jones and Hans Degens	Contributed to the design of the study, development of the experimental protocol and revised the chapter

Chapter 5:

Sarah L. Gibbings	Helped with digitizing of the capillary domain data
Hans Degens	Contributed to the design of the study and the development of the experimental protocol and revised the chapter

Chapter 6:

Richard T. Jaspers and Willem J. van der Laarse and Hans Degens

Revised the chapter and contributed to the development of the experimental protocol.

Chapter 7:

Arno F. van Heijst and Maria T. Hopman

Kindly supplied the plantaris muscles and contributed to the development of the experimental protocol.

Louis Hoofd — Helped with the model calculations of the Krogh model and revised the chapter

Richard T. Jaspers, Willem J. van der Laarse and Hans Degens

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Table of Contents

CHAPTER 1

GENERAL INTRODUCTION

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*Wüst RCI & Degens H (2007). Factors contributing to muscle wasting and dysfunction in COPD patients. International Journal of Chronic Obstructive Pulmonary Disease **2**, 289-300.*

The work described in this thesis began with an interest in the muscle problems experienced by patients with Chronic Obstructive Pulmonary Disease (COPD). For various reasons beyond our control, no patients were ever examined, but the interest persisted and led to a series of studies that may eventually throw light on the underlying physiology of this condition.

This chapter begins with an overview of COPD and the involvement of skeletal muscle in the disease and then discusses the physiology of skeletal muscle. Smoking is a major cause of COPD while tissue hypoxia may be one of the consequences that may impact on skeletal muscle structure and function. The last part of this chapter discusses the way in which the influence of these two factors on skeletal muscle function may be investigated.

Chronic obstructive pulmonary disease (COPD)

COPD is an increasingly common disease of the lungs and can be manifested as chronic bronchitis, in which the airways become irreversibly narrowed, and/or emphysema characterised by loss of elasticity of the lungs and destruction of the alveoli. Both conditions often occur simultaneously and lead to airway obstruction during expiration and an impaired gas exchange at the alveolar level. In contrast to asthma, the airflow limitation is irreversible and progressively worsens over time. Infections may accelerate the progression of the disease, and such instances are called exacerbations. Typical symptoms of the disease are shortness of breath (dyspnoea), a persistent cough, a rapid breathing rate (tachypnoea) and tiredness. The disease is progressive and is associated with an abnormal inflammatory response in the lungs to noxious gasses (ATS/ERS, 1999). In 80 % of the patients the disease is caused by smoking cigarettes for several decades but can also be caused by exposure to industrial noxious gasses and air pollution. Genetic susceptibility certainly plays a role as only about a fraction of smokers develop COPD (Molfino, 2004).

The disease is a major cause of morbidity and death throughout the world. In 2000 about 16 million people suffered from COPD in the USA alone (Mannino *et al.*, 2002) with the number of women suffering from this disorder increasing (Casaburi, 2001). The severity and progress can be moderated by actions such as smoking cessation, careful management of infections and appropriate rehabilitation (ATS/ERS, 1999).

The diagnosis of COPD involves spirometry; more specifically, measuring the forced expiratory volume in one second (FEV_1), the volume of air that can be breathed out in the first second of a maximal expiration, as well as the forced vital capacity (FVC), the greatest volume of air that can be breathed out in a maximal expiration. Normally at least 70 % of the FVC comes out in the first second (FEV_1/FVC ratio >70%), but in patients with COPD this ratio is <70 % even after medication to open up the bronchi (bronchodilators) has been given (ATS/ERS, 1999).

One of the major problems of patients with COPD is exercise intolerance (Gosker *et al.*, 2000; Casaburi, 2001). Although the disease is characterized by reduced maximal expiratory flow (ATS/ERS, 1999), the forced expiratory volume in 1 s (FEV_1) in patients with COPD correlates poorly with their exercise capacity (Killian *et al.*, 1992; Gosselink *et al.*, 1996; Engelen *et al.*, 2000; Gosker *et al.*, 2003b). Likewise, in single or double lung transplants, exercise capacity improves after surgery, but was still lower than normal (Ambrosino *et al.*, 1996) indicating that factors other than lung function alone limited exercise capacity (Evans *et al.*, 1997). Also in patients with COPD who did not undergo a lung-transplantation, evidence is growing that their exercise intolerance is not only related to a reduced lung function, but also involves skeletal muscle dysfunction (Gosselink *et al.*, 1996; Gosker *et al.*, 2003b). The importance of skeletal muscle dysfunction may increase over time as the deterioration in exercise capacity is uncoupled from the progression of airflow limitation (Oga *et al.*, 2005). It is important, therefore, to know how muscle function is affected, to identify the factors that contribute to the muscle dysfunction, including muscle wasting, in COPD so as to improve the management of the disease. Such knowledge may also have wider implications since COPD is one of a number of common disorders, including chronic heart failure (Gosker *et al.*, 2000) and some cancers (Tisdale, 2005), where muscle dysfunction and wasting are serious complications and may be brought about by similar underlying mechanisms.

Most work on skeletal muscle dysfunction and atrophy has involved patients with moderate-severe COPD, but there is a gap in our understanding of the earlier progression of the disease. As most patients with COPD have been smoking for more than a decade, it is important to understand the changes observed in smokers not yet diagnosed with COPD. A possible deterioration of muscle function and mass might then be targeted at an early stage of the disease.

Chronic obstructive pulmonary disease and muscle function

Many studies report muscle atrophy and alterations in the intrinsic properties of the lower limb muscles in patients with COPD. In most studies, the vastus lateralis muscle (part of the quadriceps muscle) was investigated and consequently this discussion will mainly focus on results obtained from this muscle. First, the adaptations will be discussed and, subsequently, various factors contributing to these changes.

Muscle atrophy

A loss of skeletal muscle mass is a common observation in patients with COPD leading to muscle weakness (Schols *et al.*, 1993; Gosselink *et al.*, 1996; Bernard *et al.*, 1998; Engelen *et al.*, 2000) and is associated with an increased mortality. Marquis and colleagues (2002) reported that 50% of their patients with a predicted FEV_1 <25 % and a mid-thigh cross-sectional area (CSA) <70 cm^2 died within 3 years, compared to only 12 % of patients with a mid-thigh CSA >70 cm^2. Schols and colleagues (1993) found that about half of the patients with mild to severe COPD had a reduced body weight, which could be related to both a loss of muscle and adipose tissue. Since lean tissue depletion could even occur in overweight patients the prevalence of muscle wasting might be even higher than the estimates based on changes in body mass alone (De Benedetto *et al.*, 2000).

Besides muscle wasting other factors, such as a decrease in maximal neural drive to the working muscles (Rutherford *et al.*, 1986), may contribute to muscle weakness during COPD. Indeed, a reduced neural drive may well explain the decline in force generating capacity per muscle cross-sectional area (specific tension) *in vivo* without a change of *in vitro* specific tension of isolated bundles from the same muscle (Debigare *et al.*, 2003). However, COPD patients who were matched for fat free mass index with control subjects did not show signs of muscle weakness or atrophy (Heijdra *et al.*, 2003; Degens *et al.*, 2005), indicating that the neural drive is maintained as long as fat free mass index is maintained.

Shift in muscle fibre type

Most studies of patients with mild to moderate COPD have shown a greater proportion of type II fibres suggesting a slow-to-fast transition in fibre type composition (Jobin *et al.*, 1998; Gosker *et al.*, 2002) as a result of the disease. The

slow-to-fast transition appears to be more marked during emphysema than during chronic bronchitis (Gosker *et al.*, 2002) and to be related to the severity of the disease in terms of FEV_1 (Satta *et al.*, 1997). It is unlikely that the changes in fibre type composition during COPD, a disease that mostly becomes manifest after the age of 50, are just a reflection of the ageing process as normal ageing is, if anything, accompanied by a fast-to-slow transition (Degens & Alway, 2006; Korhonen *et al.*, 2006). It remains to be established whether the slow-to-fast fibre type transition is sufficient to cause a change in the rates of contraction and relaxation during electrically evoked isometric tetani, as they appeared unaltered during electrically evoked tetani in patients with COPD (Degens *et al.*, 2005). As type II fibres are less efficient than type I fibres for force generation during relatively slow movements (Stienen *et al.*, 1996) and do not have a high mitochondrial density needed for ATP resynthesis, the slow-to-fast-transition in fibre type composition may be a component of the commonly observed increase in skeletal muscle fatigability (Mador *et al.*, 2003) and reduced mechanical efficiency of COPD patients during one leg knee extensor exercise (Richardson *et al.*, 2004).

Metabolism and capillarisation

Several studies have addressed the metabolic characteristics of muscles from COPD patients (Jakobsson *et al.*, 1990; Jakobsson *et al.*, 1995; Jobin *et al.*, 1998; Whittom *et al.*, 1998; Gosker *et al.*, 2002) but with equivocal results. Part of the discrepancies in the literature can be ascribed to differences in disease severity, medication and whether locomotor or other muscles have been studied. While the oxidative capacity of the vastus lateralis muscle of patients with moderate-to-severe COPD was found to be significantly reduced (Jakobsson *et al.*, 1995; Gosker *et al.*, 2002), the oxidative capacity in the musculature of the upper extremity was not affected (Gea *et al.*, 2001b). Also, the mechanical efficiency was lower in leg muscles, while arm mechanical efficiency was not significantly affected (Franssen *et al.*, 2002). The different effects on upper body and leg muscles associated with COPD were so marked that it is referred to as 'the compartment theory' (Gea *et al.*, 2001a). A simple explanation put forward for the differences between the two 'compartments' is the different degree of disuse they experience during COPD (Gosselink *et al.*, 2000; Gea *et al.*, 2001a). These results highlight the likely importance of disuse in the skeletal muscle adaptation during the progression of the disease. Nevertheless,

the glycolytic capacity was elevated in both the leg (Jakobsson *et al.*, 1995) and upper body (Gea *et al.*, 2001b) muscles. In advanced stages of the disease, energy metabolism in the quadriceps femoris muscle of patients with respiratory failure becomes increasingly compromised as reflected by lower levels of glycogen, ATP and PCr (Jobin *et al.*, 1998; Whittom *et al.*, 1998; Richardson *et al.*, 2004).

Very little is known about the capillarisation of skeletal muscle from smokers and patients with COPD (Whittom *et al.*, 1998). Although the number of capillaries per fibre has been reported to be reduced in COPD (Whittom *et al.*, 1998), the capillary supply per fibre cross-sectional area (FCSA) and total numerical capillary density was maintained (Richardson *et al.*, 2004). It seems that, at least anatomically, the capillary network is intact in COPD patients (Jobin *et al.*, 1998). However, Oga *et al.* (2007) found an almost 50 % decrease in capillary to fibre ratio and capillary density, suggesting that a disproportionate loss of capillaries. It remains to be seen to what extent these discrepant findings are related to the severity and/or medication of the disease. An alternative approach to examining the extent of muscle perfusion is to determine muscle blood flow but this is not necessarily a reliable indication of tissue oxygenation as blood flow may be maintained, passing through arterioles, and bearing a poor relationship to the critical capillary perfusion.

Skeletal muscle fatigue

Exercise intolerance, as reflected by a low peak oxygen uptake, is a major symptom in patients with COPD (Aliverti & Macklem, 2001). The increased load and consequent oxygen need of the respiratory muscles in COPD patients and reduced venous return, due to abnormal increases in abdominal pressures during breathing with expiratory flow limitations, results in a reduced cardiac output and oxygen delivery to the working muscles (Aliverti *et al.*, 2005). The result of the increased oxygen demand by the respiratory muscles and the reduction in cardiac output is an increased competition between respiratory and locomotor muscles, resulting in a imbalance between oxygen demand and supply to the locomotor muscles (Aliverti & Macklem, 2008). Under circumstances where the cardio-respiratory system is unlikely to be the limiting factor, such as during one-leg exercise, or exercise of a single muscle or muscle group, the capillarisation and oxidative capacity of a muscle are important determinants of muscle fatigue resistance, which is often reported to be

lower in patients with COPD (Serres *et al.*, 1998; Allaire *et al.*, 2004; Coronell *et al.*, 2004; Van 't Hul *et al.*, 2004; Saey *et al.*, 2005; Janaudis-Ferreira *et al.*, 2006). However, to what extent this reduction in oxidative capacity and capillarisation is the primary cause for exercise intolerance has been a recent topic of discussion (Aliverti & Macklem, 2008).

Besides changes in the muscle itself that may cause an earlier onset of muscle fatigue in COPD patients, differences in fatigue resistance could also be caused by an altered central drive. To date, the central component in the development of muscle fatigue in COPD patients is poorly understood. However, it may play a role in the development of fatigue as systemic inflammation has been shown to cause feelings of tiredness (Spath-Schwalbe *et al.,* 1998). However, muscle fatigability has been determined largely with series of voluntary contractions (Serres *et al.,* 1998; Coronell *et al.,* 2004; Janaudis-Ferreira *et al.*, 2006), which makes it difficult to differentiate between central and peripheral factors. Using electrical or magnetic stimulation, however, one can exclude the contribution of central factors to the development of fatigue. As far as we know, only two studies (Degens *et al.,* 2005; Swallow *et al.*, 2007) have assessed peripheral muscle fatigue using electrical stimulation in patients with COPD, with equivocal results. In the study of Degens and coworkers (2005), neither differences in contractile properties nor fatigability were found. This indicates that there were no apparent differences in motivation between patients and controls matched for fat free mass index and physical activity level (Degens *et al.,* 2005). Several studies have explicitly stated that the COPD patients were significantly less active than the controls (Serres *et al.,* 1998; Coronell *et al.,* 2004). Many of the skeletal muscle adaptations in COPD are also found with extreme inactivity (Gerrits *et al.*, 1999) and hence disuse may play an important role (Serres *et al.*, 1998).

A factor that has not have received much attention in the literature is the effect of smoking on skeletal muscle structure and function. As mentioned at the start of this chapter, COPD is mainly caused by cigarette smoking. Smoking is associated with a marked increase in inflammatory markers (e.g. TNF-α, IL-6) and an acute reduction in the anti-oxidant capacity (Tsuchiya *et al.*, 2002). Increases in systemic inflammation and oxidants are commonly associated with muscle atrophy (Li *et al.*, 2003; Langen *et al.*, 2006) and increased central fatigue (Robson-Ansley *et al.*,

2004). A selective atrophy of fast-twitch type IIb muscle fibres has been shown in smokers (Örlander *et al.*, 1979; Nakatani *et al.*, 2003), which is also observed in patients with moderate-to-severe COPD (Gosker *et al.*, 2003a). Others, however, found a selective atrophy of type I fibres in non-COPD smokers (Montes de Oca *et al.*, 2008). Moreover, recently Gosker and colleagues (2009) have shown that a small degree of fibre atrophy was observed and mitochondrial enzyme function was mildly impaired in mice exposed to chronic cigarette smoke. As these mice also featured clinical symptoms of COPD (emphysema), it is not clear at what stage these changes in the skeletal muscle tissue have occurred. However, few studies have looked at the skeletal muscle structure and function in otherwise healthy smokers and non-smokers.

Mild hypoxaemia may develop in patients with COPD (Chaouat *et al.*, 1997), resulting in tissue hypoxia. Mean arterial oxygen pressures of 63 mm Hg have been reported for stable patients with COPD (115 mm Hg in healthy subjects) (Chaouat *et al.*, 1997), which is similar to what would be seen in healthy people at an altitude of ~4000 m (Wagner *et al.*, 1986).

Impaired oxygen delivery, systemic inflammation and oxidative stress all seem to contribute to adaptations of skeletal muscle and the development of fatigue (for a more detailed review, see Wüst & Degens (2007) and the discussion of Aliverti & Macklem (2008)). Skeletal muscle adaptations, such as wasting and weakness, have frequently been observed with exposure to hypoxia (i.e. Green *et al.*, 1989; Ferretti *et al.*, 1990; Cerretelli, 1992; De Paula Brotto *et al.*, 2001; Deveci *et al.*, 2002). For instance, just 8 weeks at altitudes greater than 5000 m has been shown to cause as much as a 10 % reduction in muscle mass and peak power (Ferretti *et al.*, 1990; Green *et al.*, 1989). Although a decrease in fiber CSA is associated with exposure to hypoxia, other confounding factors such as decreased food intake, due to hypoxia-induced expression of leptin, together with detraining, may contribute to muscle wasting (Westerterp & Kayser, 2006). Moreover, hypoxia may also induce inflammation (Orth *et al.,* 2005), causing muscle atrophy through inflammatory pathways that may activate the ubiquitin proteosome pathway and hamper the regenerative capacity of the muscle (for more details, the reader is referred to Wüst & Degens (2007). It is therefore not surprising that hypoxaemia is thought to

contribute to the muscle adaptations observed during COPD (Gosker *et al.*, 2000; Raguso *et al.*, 2004; Wüst & Degens, 2007).

There are a variety of factors that could lead to the changes in muscle mass and function in COPD and these include the systemic consequences of the inflammatory changes in the lungs and tissue hypoxaemia occurring as a consequence of the impaired lung function (Gosker *et al.*, 2000).

Summary of skeletal muscle involvement in COPD

Although COPD is primarily a disease of the lungs there is ample evidence that skeletal muscle is also involved in many patients and this gives rise to weakness and, probably, excessive fatigue, all of which have deleterious effects on the quality of life. Smoking, which is a major cause of COPD, may have direct effects on muscle function and it is interest to know, first, whether such changes are evident before clinical symptoms of lung disease become apparent and, secondly, the possible mechanism of any such action. Tissue hypoxia is a likely consequence of severe lung disease and could lead to muscle wasting, that may be detrimental, but also changes in metabolic characteristics that could be protective.

These aspects are investigated in the chapters describing the experimental work of this thesis but before doing so it is appropriate to consider the basic physiology of muscle to see how structure is related to function and the factors that determine strength, speed and fatigability.

Structure related to function in healthy muscle

Skeletal muscle constitutes 40-50% of the healthy human body mass, and provides the means to move, communicate, feed and breathe. By any standards it is, therefore, an important tissue. Understanding its structure and function and how these may change with age, use, abuse and disease is a fascinating task and one that has engaged the scientific community for over a century. The word “muscle” comes from the Latin word *musculus*, being the diminutive of *mus* (mouse) and it is said that this came about because the movement of muscles under the skin, possibly the twitching of the biceps, resembles the movement of mice under a sheet.

The primary function of skeletal muscle is to generate force and movement and this is very dependent on the nature and physical arrangement of the contractile proteins and associated control systems.

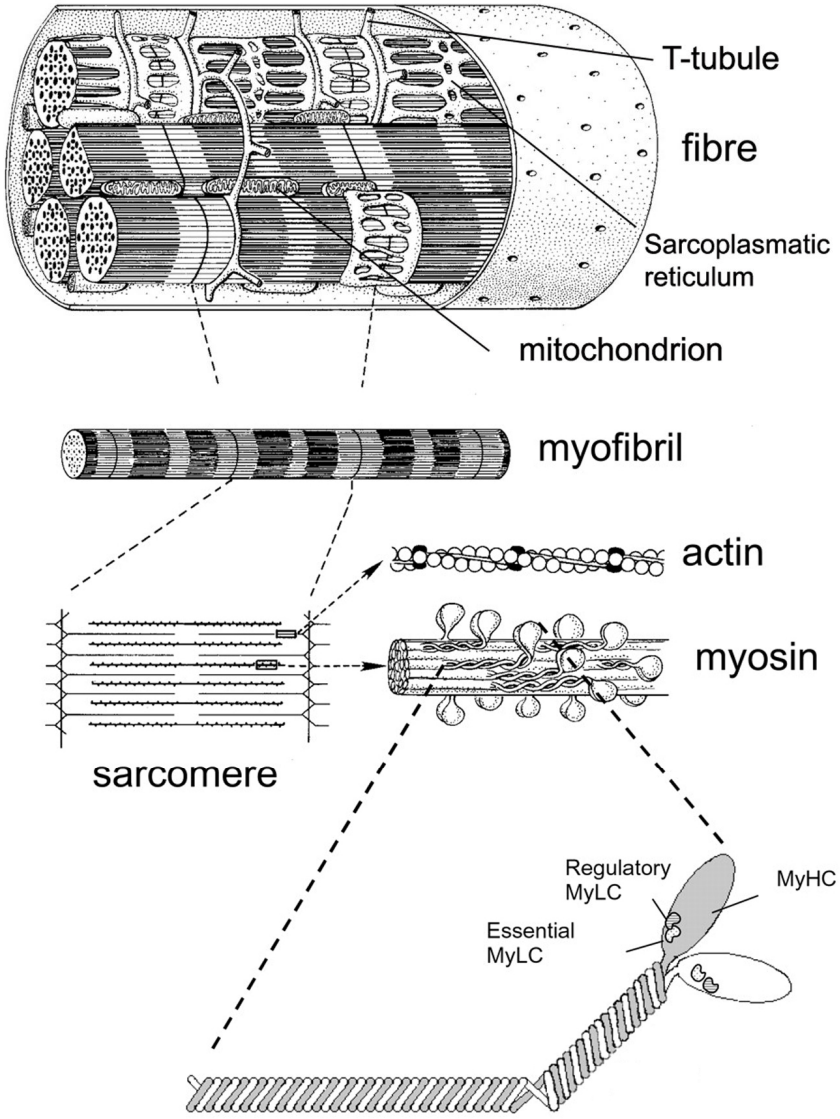

Figure 1.1. Organization of sarcomeres, mitochondria, T-tububles and sarcoplasmatic reticula within the muscle fibre. MyHC: myosin heavy chain, MyLC: myosin light chain). Adapted from Korfage *et al.* (2005).

An overview of skeletal muscle structure

Skeletal muscle consists of bundles of contractile muscle fibres that join a tendon at each end, generally running over at least one joint and thus causing a moment around that joint. The skeletal muscle fibres themselves are multinucleated cells and in mature, undamaged fibres the nuclei are located just beneath the sarcolemma. The

contractile material, which is mainly made up of interdigitating thin (actin) and thick (myosin) filaments, is arranged in sarcomeres, running from Z line, or disk, to Z line (Figure 1.1).

The contraction of skeletal muscle is controlled by the nervous system. One motor nerve (innervated by a motoneuron in the spinal cord) can branch into tens, hundreds, or even over a thousand branches, each one terminating on a different muscle fibre. One motoneuron, its axonal nerve and all of the fibres that it innervates is called a motor unit. When a motoneuron in the spinal cord is activated, an action potential is sent down the axonal braches to the neuromuscular junction (NMJ). Upon arrival at the NMJ acetylcholine is released from the axon terminal, binds to acetylcholine-receptors in the post-synaptic membrane, activating ligand-gated ion channels that allow sodium to enter and depolarize the muscle cell membrane. This depolarization of the cell membrane spreads across the surface of the muscle fibre and into the transverse tubules (T-tubules). Modified calcium channels (dihydropyridine receptors) in the T-tubules are activated in response to electrical stimulation, causing the calcium-release channels (ryanodine receptors) on the adjacent membrane of the sarcoplasmic reticulum (SR) to open. The [calcium] in the cytosol rises from ~10^{-9} M at rest to ~$10^{-4.5}$ M during maximal activation and binds to troponin C which modulates the orientation of the tropomyosin on the actin, allowing the myosin head to bind to the thin filament. Force and movement are generated by the bending of the neck region of the myosin which draws both Z lines closer to each other, shortening the muscle. This process requires utilization of adenosine-5'-triphosphate (ATP). The process from neural excitation to the activation of the thin filaments is known as excitation-contraction coupling.

An overview of skeletal muscle function

Skeletal muscle comes in a variety of colours, forms and functions. As early as 1873, Ranvier recognized that there are red and white muscles which exhibit different contractile properties and functional characteristics (Ranvier, 1873). In the second half of the twentieth century adult muscle fibres types were classified mainly according to their expressed myosin ATPase isoforms by using histochemical techniques (Brooke & Kaiser, 1970; Guth & Samaha, 1970).

Three functional output measures that are important in skeletal muscle function are strength, speed and fatigability. The requirement for strength is evident when

considering the task of simply moving the body mass or external objects, and a certain minimum strength is required to live an independent life. The force that can be exerted is mainly determined by the cross-sectional area of a muscle, that is, the amount of contractile material arranged in parallel sarcomeres. The maximal speed by which the muscle contracts depends partly on the length of the muscle but also on the intrinsic properties of the myosin heavy and light chains that affect the rate at which they interact with the actin filaments (Jones *et al.*, 2004). There is a variety of different "types" or isoforms of myosin that have been the basis for identifying different types of muscle fibre (Sant'ana Pereira *et al.*, 1995; Pette & Staron, 2000). This variety in muscle fibre type is the major link between structure and function (Harridge *et al.*, 1996; Pette & Staron, 2000).

Muscle power is the product of force and speed of the contraction. Since work output is the main feature of movement and mobility, the power that can be generated by a muscle has a major impact on physical activity, whether this is in a sporting context or determining the extent of an independent life of an elderly person or patient. Some tasks, such as jumping or throwing require only brief efforts but many activities associated with mobility, and therefore quality of life, require sustained efforts. In these circumstances the ability of the muscle to withstand the development of fatigue is important. Resistance to fatigue varies between different types of muscle fibres and is affected by factors such as use and disuse.

Clearly, limitations in power generating capacity and an excessive loss of fatigue resistance of the muscle can seriously limit performance and daily activities, and these changes in muscle function can become critical in various disease states such as cancer, HIV and COPD.

Skeletal muscle fibres

Skeletal muscle fibre types

To identify the different types of myosin ATPase in muscle sections by histological means, they are pre-incubated at either an alkaline or acid pH. Although the values vary somewhat from species to species, in general, fast myosin ATPase is unstable and inactivated at acid pH (about pH <4.6) while slow myosin ATPase is inactivated at a pH>9.4. Having pre-incubated the section and inactivated the ATPase activity in

one set of fibres, the remaining activity can be visualised by incubating the section with ATP and an excess of calcium at an alkaline pH. After a couple of chemical steps that convert inorganic phosphate, the product of the ATPase reaction, into a black or dark brown precipitate, the fibres with remaining myosin ATPase activity can be identified (Brooke & Kaiser, 1970; Guth & Samaha, 1970). An example of this staining method is given in Figure 1.2A.

Based on these ATPase staining methods, developed in the 1960s, muscle fibres in all species were divided into type I, IIa and IIb. These types are a reflection of the type of myosin heavy chain (MyHC) within the fibre with type I, IIa and IIb fibres containing type I, IIa and IIb myosin heavy chain, respectively. Twenty years later it

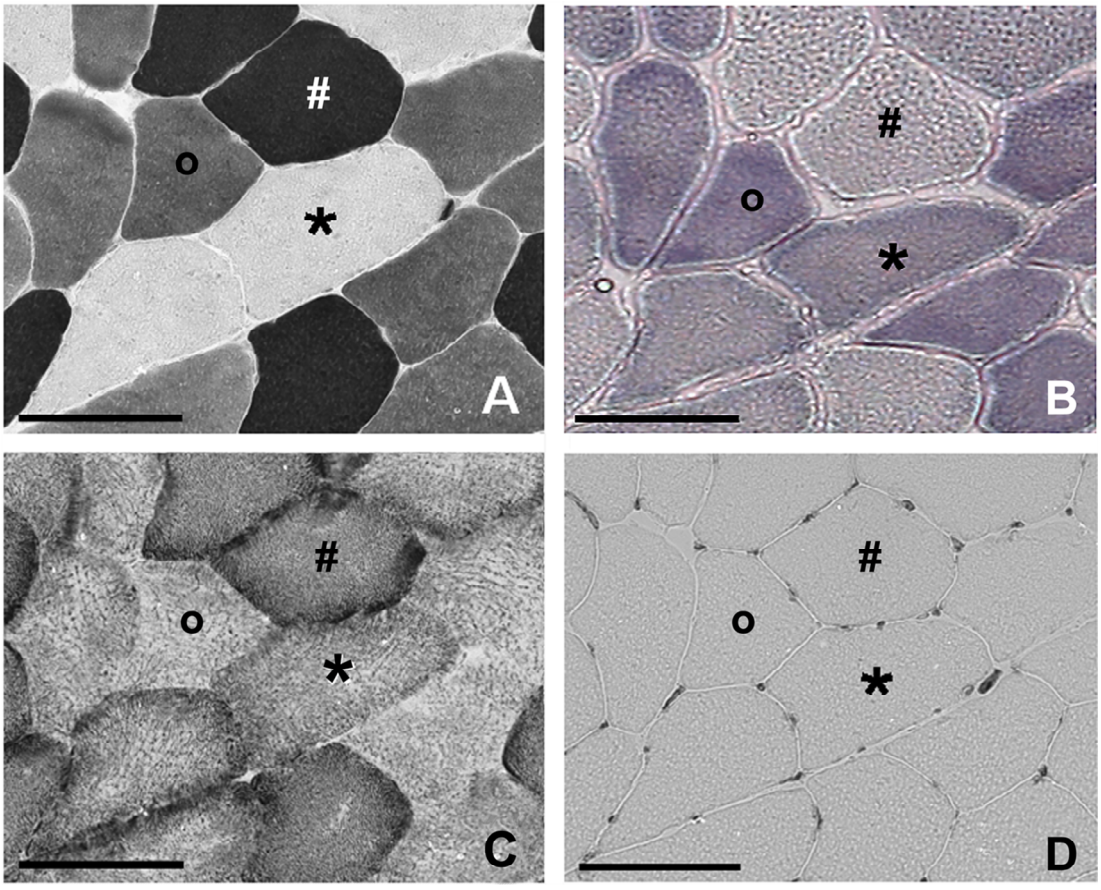

Figure 1.2. Serial sections of the human vastus lateralis muscle stained for **A** myofibrillar ATPase (mATPase) according to Brooks and Kaiser (1970) (preincubation at pH 4.55), with black fibres representing type I, white are type IIa and grey are IIx fibres, **B** cytosolic glycerol-3-phosphate dehydrogenase, as a marker for glycolytic capacity, **C** succinate dehydrogenase (SDH) - activity, an enzyme bound to the inner membrane of the mitochondria and **D** capillaries (lectin). Sections taken from a biopsy given by a young male participant described in Chapter 6.
#,* and o depict a type I, IIa and IIx fibre respectively.

was shown that there is also an intermediate type, sometimes known as IId (Termin *et al.*, 1989) but more commonly referred to as IIx (Larsson et al., 1991). Subsequently it was shown that for human muscle, what had been classified as IIb was similar to rat IIx myosin (Pereira Sant'Ana *et al.*, 1997) and it appears that human muscles do not express the IIb MyHC isoform found in smaller mammalian species. Histochemical techniques generally demonstrate just one myosin isoform in a fibre cross-section. However, electrophoretic separation of MyHC from single muscle fibres or immunohistochemical staining show that while the majority of fibres express just one isoform, they can express two, and occasionally more, MyHC isoforms (Sant'ana Pereira *et al.*, 1995; Yu *et al.*, 1998).

Glycolytic capacity

It is possible to visualise a number of enzyme activities as indicators of glycolytic potential, one commonly used being glycerol-3-phosphate dehydrogenase (GPDH) (also known as alpha-glycerol phosphate dehydrogenase). Although this enzyme is not part of the chain of glycolysis, it is related to the glycolytic pathway because it shuttles NADH into the mitochondria. An example of this staining method is given in Figure 1.2B.

Mitochondrial enzyme activities

Staining for mitochondrial activity requires a substrate that is oxidised and an electron acceptor that is reduced by one of the components of the electron transport chain and, as a result, changes colour. Using succinate as substrate and tetrazolium blue as the electron acceptor gives a measure of complex II activity (succinate dehydrogenase, SDH, Pool *et al.*, 1979a; Pool *et al.*, 1979b). The measured activity is proportional to the mitochondrial density and the integrated SDH activity (SDH x FCSA) is linearly related with the maximal oxygen uptake of the cell (in nmol mm^{-3} s^{-1}, Van der Laarse 1989). An example of this staining method is given in Figure 1.2C.

Capillary supply

Ultimately, oxygen is used during oxidative phosphorylation to generate ATP from NADH and $FADH_2$ generated during glycolysis, fatty acid oxidation and the citric acid cycle. For this process to take place an adequate supply of oxygen is required

and heat, water and carbon dioxide (CO_2), being waste products from this process, have to be removed. The exchange of oxygen, heat and waste products between the blood and tissue takes place in the capillaries. It is thus not surprising that the capillary supply, in terms of capillary density, to a muscle fibre is higher for oxidative than glycolytic fibres (Degens *et al.*, 1992) and that capillary recruitment and blood flow varies with the level of activity of the muscle fibres (Terjung & Engbretson, 1988). Given these conditions, it is likely that the capillary supply of the muscle plays a significant role in the resistance to fatigue, as has been observed in control and chronically stimulated muscles (Hudlicka *et al.*, 1977). Capillaries can be visualized in muscle cross-sections by histological stains, e.g. alkaline phosphatase, or lectin (Figure 1.2D). The capillary density of skeletal muscle tissue is usually expressed as the number of capillaries per unit of tissue, or the capillary to fibre ratio. From these measures, indications of tissue oxygenation can be obtained by modelling oxygen transport from the capillaries to the centre of the muscle fibres (i.e. Krogh and Hill models, Krogh (1919); Hill (1965)). There is an apparent relation between capillary density and maximal blood flow within a muscle, but flow is primarily determined by arterioles and not capillaries (Egginton, 2009).

Contractile properties

In general, type I fibres predominate in muscles which have a red appearance and, as Ranvier observed, are slow contracting. Pale "white" muscles are fast contracting (Ranvier, 1873) and contain a predominance of type II fibres. Muscle fibres expressing mainly type I MyHC are characterised by having slow speeds of contraction and relaxation. They also tend to be fatigue resistant and have a relatively high activity of oxidative enzymes. On the other hand, type II fibres have short contraction and relaxation times, they fatigue more rapidly and mostly rely on glycolysis to obtain their energy during activity.

In mammals the soleus is an example of a red muscle that is composed mainly of type I fibres while the extensor digitorum longus (EDL) is pale in appearance and has mainly type II fibres. The soleus and EDL are extreme examples; most muscles contain a mixture of different fibre types. Although mammalian skeletal muscles all show similar varieties of fibres when classified by their histochemical properties, there are considerable differences between species as regards contractile characteristics.

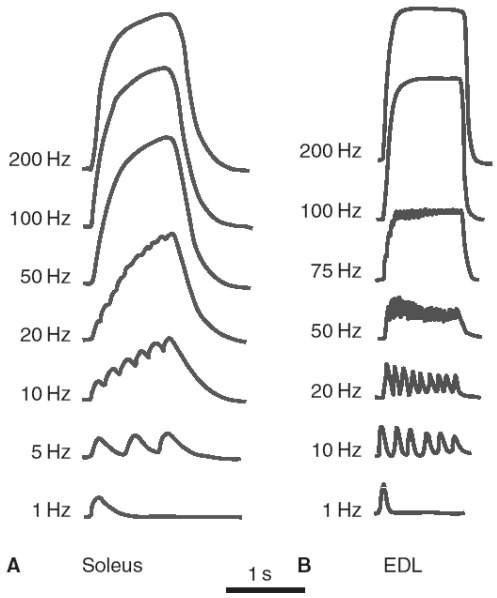

Figure 1.3. Example of force generated during stimulations with different frequencies in isolated mouse from **A** soleus and **B** extensor digitorum longus muscles (25 °C). From Jones *et al.* (2004).

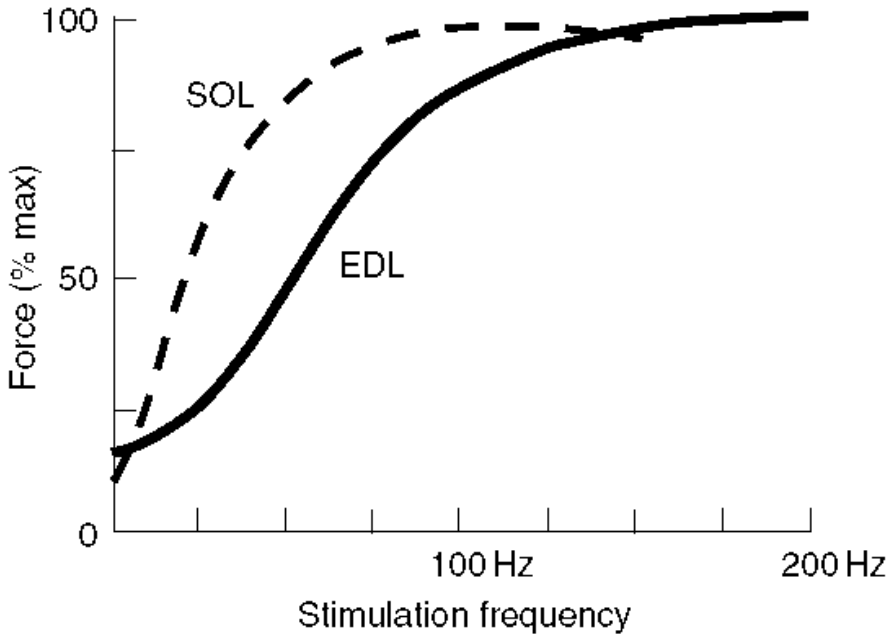

Figure 1.4. Relationship between force and stimulation frequency in isolated mouse soleus and extensor digitorum longus (EDL) muscle. Note the left-shift in the muscle with predominantly type I fibres. From Jones *et al.* (2004).

The time it takes for the muscle to achieve its maximal force (contraction time) and the time to fully relax (relaxation time) are both much faster in the EDL compared to the soleus muscle (see Figure 1.3). At a stimulation frequency of 10-20 Hz, the EDL

muscle relaxes fast enough for the force to fall back to baseline before the next impulse. However, for the soleus muscle, the next impulse comes before total relaxation of the previous twitch is complete and the next contraction is superimposed on the remaining force. In this way, the individual twitches fuse. The fusion frequency at which a smooth contraction (tetanic contraction) is generated is therefore higher in fast-twitch, compared to slow-twitch fibres and as a result, the force-frequency relationship between these two muscle types differs (Figure 1.4).

The characteristics of a skeletal muscle are strongly related to its functional demands (Flück & Hoppeler, 2003). Depending on the altered environmental conditions, specific muscular adaptations occur as a result of increased use, such as an increase in oxidative capacity in response to endurance training or hypertrophy after resistance training (Hudlicka *et al.*, 1977; Holloszy & Coyle, 1984), atrophy after chronic disuse (Larsson *et al.*, 1990; De Boer *et al.*, 2007) and disease (De Haan *et al.*, 2000; Gosker *et al.*, 2000; Debigare *et al.*, 2003), but also environmental conditions such as hypoxia where muscle atrophy and loss of oxidative capacity have been observed (Ferretti *et al.*, 1990; Green, 1992).

The mechanisms underlying differences in contractile properties

The difference in contractile properties between fibre types is mainly attributable to differences in MyHC composition (Harridge *et al.*, 1996), such that the maximal speed of shortening of the fibres is in the order MyHC I << MyHC IIa<MyHC IIx<MyHC IIb (Bottinelli *et al.*, 1996). However, factors that control Ca^{2+} flow and activation also play a role in the contractile speed and fatigability of the muscle fibre (Allen *et al.*, 2008b). When a muscle action potential is initiated at the NMJ, the action potential transmission is conducted down the T-tubules to the voltage sensors in the T-tubular membrane. Type II fibres have a higher density of dihydropyridine receptors (Hollingworth & Marshall, 1981), a 50% times higher density of the SR (Cullen *et al.*, 1984) and a higher density of ryanodine receptors (Franzini-Armstrong *et al.*, 1988). Consequently the release of Ca^{2+} from the SR into the cytoplasm is greater and faster for fast-twitch compared to slow-twitch fibres (Briggs *et al.*, 1977). Moreover, the isoform of troponin in type II fibres contains two Ca^{2+}-binding sites, rather than one for the slow type troponin isoform (Schiaffino & Reggiani, 1996).

The SR Ca^{2+}-pump expressed in fast fibre type (SERCA1) is faster compared to the isoform found in type I fibres (confusingly called SERCA2). These processes involved in the activation and deactivation of the contractile elements in different fibre types correspond with the rate of cross-bridge interaction and account for the differences in contractile properties such as illustrated in Figures 1.3 and 1.4.

The higher attachment and detachment rates of the type II myosin cross-bridges leads to faster rates of shortening and higher power output but this advantage comes at the expense of a higher ATP consumption per cross-bridge cycle (Stienen *et al.*, 1996). The 6-fold larger density of SR Ca^{2+} pumps in the type II fibres (Everts *et al.*, 1989) results in much quicker reuptake of Ca^{2+} in the SR, but also at a higher cost of ATP. These two processes mean that ATP turnover is considerably higher in fast fibres and leads to a more rapid depletion of intracellular energy stores if not met by a commensurate higher energy production. The balance between rates of energy utilization and production has a major impact on the rate at which muscle fibres fatigue.

Muscle fatigue

Fatigue is a common phenomenon experienced in daily life and during exercise. It may come about for a variety of reasons and has a number of consequences for muscle function. Studies of muscle fatigue in the literature vary with regard to the exercise model, protocol, output variables and definitions for fatigue, so that comparison of the results is often difficult. Some studies focus on functional aspects, such as changes in running speed; others concentrate on specific aspects of changes in function of single muscles or muscle fibres, or changes in metabolite concentrations. Vøllestad (1997) defined fatigue as: '*any contraction-induced reduction in the capacity to generate force*' although this could be amended to include changes in contractile speed that have consequences for power output. It might also include the stipulation that the changes should be reversible within a "reasonable" time frame to distinguish fatigue from damage that can also result from prolonged activity.

The generation of force or movement involves a long chain of command originating in the motivation of the individual, involving the motor cortex, the descending neural pathways, the processes of excitation-contraction coupling and ending with the interaction of the actin and myosin cross bridges. Therefore, at least in theory, a reduction in the capacity to generate force could be the result of an impairment of any of the factors mentioned above. Fatigue due to factors proximal to the neuromuscular junction is conventionally called "central" fatigue while fatigue occurring at, or distal, to the neuromuscular junction is called "peripheral" fatigue. Although causes of failure at these two sites are generally site specific they may sometimes affect multiple steps in the chain. For instance, fatigue due to a lack of oxygen supply could affect central processes (cerebral hypoxia), or peripheral (referring to the impaired energy metabolism in the muscle fibre) fatigue.

Central fatigue

Central fatigue is defined as a progressive reduction in the voluntary activation of the muscle during exercise (see review by Gandevia, 2001). During a voluntary contraction the central nervous system may not maximally activate all the motoneurons of an individual muscle and this proportion may decrease during prolonged activity as a consequence of afferent feedback from mechano-, chemo- and/or thermo-receptors activated by the physical activity (Bigland-Ritchie *et al.*, 1978) or as a consequence of increased activity of inhibitory circuits within the brain itself. An inability to voluntarily contract all the muscle fibres can be assessed by comparing the voluntary contraction with one elicited by electrical stimulation, usually of the peripheral nerve branches, which removes any contribution of the central nervous system (Merton, 1954; Edwards *et al.*, 1977; Bigland-Ritchie *et al.*, 1978; Rutherford *et al.*, 1986; Newman *et al.*, 2003).

Peripheral fatigue

Fatigue resulting from changes at or distal to the neuromuscular junction is known as peripheral fatigue. Failure of the neuromuscular junction (exhausting stores of neurotransmitter) does not seem to occur in maximal isometric contractions of up to 60 s (Bigland-Ritchie *et al.*, 1982) or during electrically evoked contractions at optimum muscle length with a frequency of 30 Hz (Sacco *et al.*, 1994). Once an action potential is successfully transmitted across the neuromuscular junction, it

propagates along the surface membrane of the muscle fibre into the T-tubular system leading to the interior of the fibre. At high stimulation frequencies (80-100 Hz in human muscle) failure of the membrane propagation occurs already after a few seconds (Jones *et al.*, 1979), possibly due to a build up of a high $[K^+]$ in the T-tubules, a phenomenon sometimes referred to as 'high-frequency' fatigue (Jones, 1996). However, this is thought not to be a serious problem *in vivo* as the initial voluntary firing rate is around 30 Hz during sustained contractions, which then declines as the contraction continues (Bigland-Ritchie *et al.*, 1986).

A reduction in the calcium release from the SR can contribute to the development of fatigue (Allen *et al.*, 2008a). The application of caffeine, which opens the ryanodine receptors in the SR membrane, to a fatigued muscle fibre restores most of the loss of isometric force and provides strong evidence of an impaired Ca^{2+} release from the SR, probably as a consequence of phosphate accumulating in the SR and chelating the calcium (Fryer & Stephenson, 1996). While reductions in [ATP] do not appear to play a significant role in the development of fatigue, increased concentrations of inorganic phosphate (Pi), as a result of PCr breakdown, inhibit force production by direct actions on the cross bridge function, through an impaired release of Pi from the actomyosin complex during the force-generating step of the cross bridge cycle, resulting in a reduction in tetanic force at a given pCa (Martyn & Gordon, 1992). During prolonged exercise, lasting several hours, ATP resynthesis may become limited as a result of glycogen depletion within the muscle. Although ATP is critical for muscle contraction, there is little evidence of major changes in ATP levels in fatigue. Nevertheless in situations where ATP synthesis is limited there are major changes in the concentration of associated metabolites, PCr, Pi, ADP, AMP and lactate, and it is likely that these are the critical factors giving rise to the altered contractile function that characterise fatigue.

Fatigued muscle also demonstrates (1) slowing of contractile properties, evident as a decrease in both rates of contraction and relaxation, (2) reductions in the maximal shortening velocity and (3) reductions in power output during loaded contractions (Jones *et al.*, 2006). Relaxation of the skeletal muscle fibre involves a set of steps: 1) SR Ca^{2+} release stops, 2) Ca^{2+} is pumped back into the SR, 3) Ca^{2+} dissociates from the troponin and 4) detachment of cross-bridges (Allen *et al.*, 2008a). All four steps could be involved in the slowing of relaxation during fatigue *in vivo* although there is little evidence that the kinetics of calcium release or re-accumulation are seriously

affected. There is, however, clear evidence that cross bridge kinetics are affected by fatigue (de Haan *et al.*, 1989). More recently, Jones and colleagues (Jones *et al.*, 2006) showed in healthy individuals, a strong relationship between an increased curvature of the force-velocity relationship (a/P_0) and the slowed relaxation *in vivo*, indicating that altered cross-bridge kinetics contributes to the slowing of relaxation in fatigue. As the shape of the force-velocity curve, given by a/P_0, is one of the main determinants of the power output of muscle (Sargeant, 2007), a decrease in a/P_0, indicating an increased curvature, during fatigue greatly reduces power (Jones *et al.*, 2006).

Electrical stimulation

In the studies described in this thesis, brief electrically evoked contractions have been used to test muscle function as opposed to voluntary contractions of the quadriceps muscle (see Figure 1.5 for an example). Electrical stimulation of the quadriceps muscle can be achieved by either direct stimulation of the femoral nerve, or percutaneous stimulation of the motor nerve branches within the muscle belly.

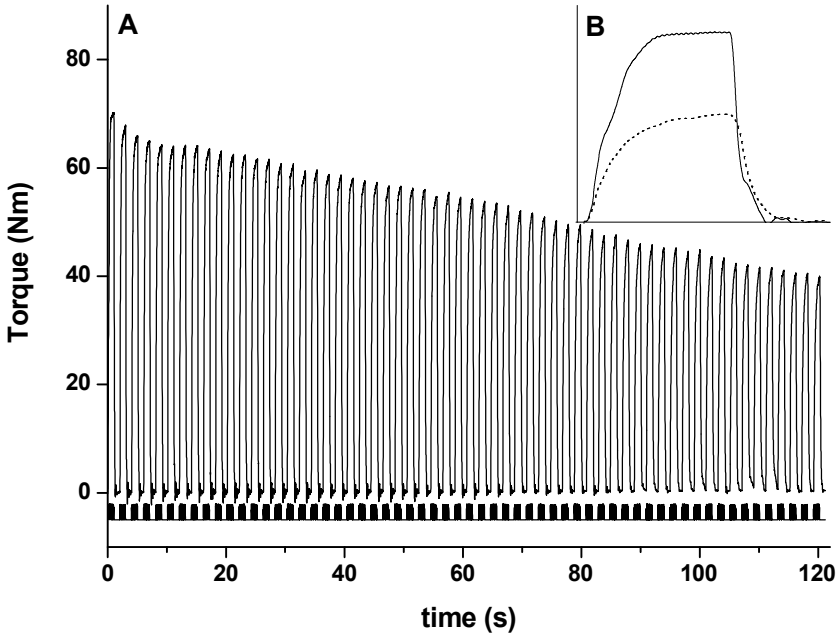

Figure 1.5. Example of the fatigue test with electrically-evoked contractions applied in this study (**A**), with in **B** the first (continuous line) and last (dotted line) contraction enlarged. Features of a post fatigued contraction are the reduced maximal force and prolonged contraction and relaxation times.

Although percutaneous stimulation of the quadriceps muscle recruits particularly motor nerve branches (and subsequently muscle fibres) in the superficial parts of the

muscle, deeper areas are also activated (Adams *et al.*, 1993). A fatigue protocol in which the muscle is activated by electrical stimulation eliminates any change in force generating capacity due to central factors. Although electrical stimulation of this type activates between 30-50 % of the muscle the recruitment pattern during electrical stimulation may differ from that during submaximal voluntary contractions of a similar strength in that the electrical stimulation will tend to recruit a slightly greater proportion of fast motor units. The stimulated fatigue test is therefore similar to a series of repeated maximal contractions.

Fatigue resistance during protocols such as illustrated in Figure 1.5 is mainly determined by the interplay of two factors, namely energy utilisation and energy production of the fibre, that is, the differences between ATP utilisation and ATP re-synthesis, although, as explained above, it is probably the levels of the associated metabolites that actually affect contractile function. Type I muscle fibres require less ATP for a given contraction at a low speed and are therefore more efficient compared to the type II fibres. During any prolonged period of activity, the re-synthesis of ATP depends on aerobic metabolism, and for this the mitochondrial density and oxygen supply to the muscle are key factors.

Aim and objectives of the thesis

It can be concluded from the work discussed above that, in general, patients with mild-to-moderate COPD have lower muscle mass and strength and experience an earlier onset of fatigue during daily life activities. Since smoking is the principle cause of COPD and many patients with COPD suffer from hypoxaemia during exercise, and often at rest, the main objective of the present thesis was to obtain a better understanding of the effects of cigarette smoking and systemic hypoxaemia on skeletal muscle structure and function with an emphasis on fatigue resistance.

To this end, smokers were studied who did not suffer from clinical signs of COPD. To minimise bias related to potential differences in physical activity level between smokers and non-smokers, special care was taken to match the smoking and control groups with regard to physical activity level.

To investigate the effect of systemic hypoxaemia on skeletal muscle morphology and skeletal muscle oxygenation, Wistar rats were exposed to hypobaric hypoxia (410 mm Hg, equivalent to ~5000 m). After 4 weeks exposure, muscle morphology was

investigated and the data used to estimate the skeletal muscle tissue oxygenation with mathematical models (Krogh, 1919; Hill, 1965).

In **Chapter 2** a fatigue protocol using percutaneous muscle stimulation (with and without ischaemia) was used to assess the peripheral muscle fatigue resistance in healthy individuals, with special relevance to sex differences in muscle fatigability. The outcomes of the fatigue protocol were related to muscle size and contractile properties.

Young smokers and their peers underwent the same protocol for the measurement of maximal strength, contractile properties and fatigue to study the effect of smoking on muscle function in young, healthy male smokers. The results and discussion of this study are presented in **Chapter 3**.

As muscle strength and fatigue resistance might deteriorate faster over time in smokers compared to non-smokers, the results from a cross-sectional study on the relationship between smoking history and muscle function are shown in **Chapter 4**.

In **Chapter 5** results that link capillarisation (and hence oxygen supply) to muscle fibre size, type and oxidative capacity are presented. Moreover, the differences in capillarity and oxidative capacity between human biopsies and animal models are shown. Histological data from smokers and non-smokers using similar methodology are compared in a study presented in **Chapter 6** of this thesis.

In **Chapter 7** changes in fibre size, oxidative capacity and capillarisation in the plantaris muscle of Wistar rats exposed to 4 weeks of chronic hypoxia are presented.

In the general discussion in **Chapter 8** the main results are summarised and discussed, suggestions for future work are given and final conclusions are drawn.

An extended version of the methods described in each chapter can be found in the **Appendix**.

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CHAPTER 2

SEX DIFFERENCES IN CONTRACTILE PROPERTIES AND FATIGUE RESISTANCE OF HUMAN SKELETAL MUSCLE

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Abstract

To explore the cause of higher skeletal muscle fatigue resistance in women than men, we used electrically evoked contractions (1 s on 1 s off, 30 Hz, 2 min), which circumvents motivational bias and allows examination of contractile properties. We compared 29 men (26.5 ± 7.0 yr, mean ± SD) with 35 women (25.4 ± 7.6 yr). Strength of the quadriceps muscle was higher in men than women ($P<0.001$). The lower maximal rate of relaxation in women ($P=0.002$) indicates that their muscles were slower than those of men. The torque declined less in women than in men (37.7 ± 10.7 % *vs.* 29.9 ± 10.0 %; $P=0.002$), and was not related to muscle strength or size, as determined with MRI. The sex-difference in fatigability was also seen when the circulation to the leg was occluded (torque declined 76.9 ± 10.8 *vs.* 59.5 ± 16.9 % in men *vs.* women respectively; $P=0.008$). The maximal rate of relaxation correlated with the fatigability of the muscle under all conditions (correlations ranging from 0.34 to 0.51, $P<0.02$). We conclude that the sex-related difference in skeletal muscle fatigue resistance is not explicable by differences in motivation, muscle size, oxidative capacity and/or blood flow between sexes, but might be related to differences in fibre type composition.

Introduction

Whilst men have, on average, bigger muscles and hence are stronger than women there is accumulating evidence that women can sustain continuous, as well as intermittent, muscle contractions at similar relative low to moderate intensities better than men (Fulco *et al.*, 1999; Russ & Kent-Braun, 2003; Hunter *et al.*, 2004a; Hunter *et al.*, 2004b; Russ *et al.*, 2005; Hunter *et al.*, 2006b). There are many possible causes for this phenomenon and, broadly, they can be summarised as 1) differences in motivation and the ability to sustain central drive, 2) differences in blood supply to the working muscles, and 3) intrinsic differences in the composition and fatigue characteristics of the fibres making up the muscle.

The possibility that has received most attention concerns the fact that during a contraction the blood supply to the working muscle will be occluded to some degree and the extent of this will depend on the forces developed within the muscle (Barcroft & Millen, 1939; Sadamoto *et al.*, 1983). A large male muscle working at the same percentage of maximum as a smaller female muscle will generate more force, occlude the circulation to a greater extent and, consequently, fatigue more rapidly during sustained submaximal contractions. The absence of sex-related differences in fatigability during sustained and intermittent volitional contractions under ischaemic conditions (Russ & Kent-Braun, 2003; Clark *et al.*, 2005), where blood flow is no longer a factor, and the observation that during sustained contractions the time to fatigue is a function of absolute force (Hunter & Enoka, 2001; Hunter *et al.*, 2006b) support this view. On the other hand the increased fatigability in men during intermittent submaximal isometric contractions is not dependent on maximal strength (Hunter *et al.*, 2004b; Gonzales & Scheuermann, 2006) and even when matched for absolute strength, men were more fatigable than women during both intermittent and sustained contractions (Fulco *et al.*, 1999; Hunter *et al.*, 2004b; Gonzales & Scheuermann, 2006). Consequently there remains uncertainty as to whether differences in blood flow to the working muscles can explain the differences in fatigability of men and women.

Whether a muscle fibre fatigues or not will depend largely on the balance between the rate of energy consumption associated with the contractile activity and the rate at

which ATP can be regenerated by anaerobic (i.e. ATP generated by hydrolysis of creatine phosphate and glycolysis) and aerobic (i.e. ATP generated by oxidative phosporylation) processes. The various muscle fibre types differ, with the faster fibres having greater energy demands but lesser capacity for oxidative phosphorylation compared with the slower fibre types. Therefore, the proportions of the different fibre types in a muscle will affect its fatigue characteristics. Several studies have reported higher muscle glycolytic enzyme activities (Jaworowski *et al.*, 2002; Russ *et al.*, 2005) and lower oxidative capacity (Nygaard, 1981; Green *et al.*, 1984) in men compared to women, which is consistent with a lower proportion of slow, fatigue-resistant, type I fibres in men (Nygaard, 1981; Carter *et al.*, 2001; Roepstorff *et al.*, 2006).

Little attention has been paid to differences in central drive. Recently, however, it was shown with superimposing stimulation that sex-related differences in fatigue during a series of sustained maximum voluntary isometric contractions were not related to differences in voluntary activation but were likely to have been of peripheral origin (Hunter *et al.*, 2006a). Surprisingly, there appears to be no studies in which muscles have been fatigued by the use of electrical stimulation, an experimental situation that completely removes the volitional element and allows a direct comparison of the extent of peripheral fatigue in men and women.

Our primary objective, therefore, was to expand on the work of Hunter and colleagues (2006a) by using electrical stimulation to directly determine whether there are differences in susceptibility to peripheral fatigue in men and women. Our second objective was to determine whether any differences in fatigability during an intermittent protocol could be attributed to differences in blood supply to the muscle. We have done this by comparing the extent of fatigue seen with an intact circulation with an identical fatigue protocol carried out under ischaemic conditions. The hypothesis was that if the differences are attributed to differences in blood supply or oxidative capacity of the muscle then no sex differences will be evident when the blood supply is occluded.

Methods

Participants

Sixty four volunteers (29 men, 35 women) aged between 19 and 45 years participated in the study. Men and women were matched for age and physical activity, the latter assessed by questionnaire (Baecke *et al.*, 1982). Exclusion criteria were smokers, known respiratory, cardiovascular or neuromuscular diseases, and any lower limb injury. Written informed consent was obtained from each participant prior to testing. The study was approved by the local Ethical Committee to the standard set by the Declaration of Helsinki.

Experimental procedures

Muscle size

Anatomical cross-sectional area (ACSA) of the quadriceps muscle was measured at 50% femur length with magnetic resonance imaging (MRI), using a fixed 0.2-T MRI-scanner (E-scan, ESAOTE Biomedica, Genova, Italy). Scans were obtained with a T1-weighted, high resolution, gradient echo profile, with the following scanning parameters: echo time: 0.016 s; repetition time: 0.1 s; field of view: 0.33 x 0.254 m; matrix: 256 x 256 and a slice thickness: 0.005 m.

Torque measurements

All torque measurements were performed on the right quadriceps femoris muscle with a Cybex norm dynamometer (Ronkonkoma, New York, USA). Participants were familiarised with the testing procedures on a separate occasion prior to data collection. Participants were seated with the hips (90° flexion) and shoulders strapped to minimise extraneous movements. The participants received visual feedback of the torque signal and verbal encouragement during the maximal voluntary contractions (MVC). Maximum voluntary contractions were repeated 2 or 3 times at knee joint angles of 60, 70 and 80˚ (full extension=0˚) in a random order with two minutes of rest in between each contraction to prevent development of fatigue. The angle at which the highest torque was achieved was defined as the optimal joint angle. All further measurements were done at optimal knee joint angle. To account for differences in muscle cross-sectional area between the two groups,

maximal knee extensor torque was scaled with quadriceps ACSA (Maughan *et al.*, 1983) and expressed as MVC/ACSA (Nm cm^{-2}).

Electrical stimulation

Percutaneous electrical stimulation (square wave, pulse width 50 μs; DSV Digitimer Stimulator, Digitimer Ltd., Herts, UK) was applied using carbon-rubber pads (76 mm x 127 mm, Versastim, Conmed Corp., N.Y., USA). The anode was placed over the proximal region of the quadriceps and the cathode over the distal third of the upper leg.

Voluntary activation (VA)

To assess the ability of the subjects to activate the knee extensors during isometric contractions, voluntary activation levels were determined using a variant of the interpolated twitch technique (Shield & Zhou, 2004). For the test, a doublet (inter-pulse interval 10 ms) was applied with the subject in a relaxed state and a second interpolated doublet was delivered during the plateau phase of the MVC. The current used to test for activation was determined using single pulses with the subject in a relaxed state, starting from 100 mA, and increasing until no further increase in torque was observed. Throughout the experiments, the voltage was kept at 400 V.

Torque-frequency relationship

The intensity of stimulation for the determination of the torque-frequency relationship and fatigue protocols was adjusted so that a 1 s 100 Hz tetanus produced 28.6 ± 6.7 (SD) % of maximal voluntary isometric torque. Five minutes after the last MVC, the quadriceps muscle was stimulated with 1 s 1, 10, 15, 20, 30, 50 and 100 Hz trains in random order, each separated by 1 min to assess the torque-frequency relationship.

Fatigue tests

Five minutes after determining the torque-frequency relationship of the quadriceps muscle, the resistance to fatigue was determined by a series of electrically evoked isometric contractions (60 contractions, 30 Hz, 1 s on 1 s off; the standard fatigue test)

On two subsequent occasions, a subgroup of subjects also performed two more fatigue tests that were metabolically more demanding. In the first the rest period was

reduced from 1 s to 0.5 s, while leaving the total duration of the test the same (12 men and 18 women). In the second test the blood supply to the muscle was occluded with a pneumatic thigh cuff (Accoson, Harlow, UK) inflated to ~240 mm Hg before and during the standard fatigue protocol (12 men and 10 women). The occlusion prevented the return of blood in the intervals between the stimulated contractions and hence oxidative recovery (Russ *et al.*, 2002).

Analysis of variables

The torque signal was stored with a sampling frequency of 2000 Hz (Acknowledge, Biopac Systems, Santa Barbara, CA, USA) and off-line analysis was performed using Matlab (Matlab, the Mathwork Inc., S. Natik, MA, USA). Each torque signal was filtered with a low-pass fourth order Butterworth filter with a 30 Hz cut-off frequency. The maximal rate of relaxation (MRR, s^{-1}) was determined from the 30 Hz tetanus and calculated as the lowest value of the differentiated torque signal expressed relative to the highest torque recorded during that contraction.

Statistical analysis

Differences in MVC, ACSA and MRR between the sexes were tested using an independent Student's t-test for all dependent variables. Voluntary activation levels were analysed with the non-parametric Mann-Whitney U test, because the values were not normally distributed. Differences were considered significant at an alpha of 0.05. Results are presented as means (SD), unless otherwise stated. Torque data from the three fatigue tests and torque-frequency relationships were analyzed using repeated measures ANOVA with time and frequency, respectively, as within factor, and as between-subject variable sex.

Results

Subject characteristics, muscle strength and size

Mean physical activity scores were [mean ± SD (range)]: 8.5 ± 1.3 (6.0– 0.8) *vs.* 8.5 ± 1.0 (6.0–10.4) in men and women, respectively, indicating that we successfully matched the sexes for physical activity level. A physical activity score < 6 indicates a sedentary life-style, whereas values > 9 represent a high level of activity (Baecke *et al.*, 1982). Mean age was 26.5 ± 7.0 (20–45) *vs.* 25.4 ± 7.6 (19-45) years in men and

Table 2.1. Quadriceps muscle properties.

	Men (n=29)	Women (n=35)
ACSA (cm^2)	75.5 ± 10	55.6 ± 6.9 *
MVC (Nm)	339 ± 82	255 ± 51 *
MVC/ACSA (Nm cm^{-2})	4.50 ± 0.18	4.34 ± 0.15
VA (%)	93.4 ± 9.2	90.1 ± 5.3
Optimal angle (°)	75 ± 7	71 ± 9
MRR_{30} (s^{-1})	-15.6 ± 2.4	-14.1 ± 1.8 *

ACSA: anatomical cross-sectional area; MVC: maximal voluntary contraction; VA: voluntary activation; MRR_{30}: maximal rate of relaxation from the 30 Hz stimulus, normalised to maximal torque during that contraction; *: different from men at $P<0.01$.

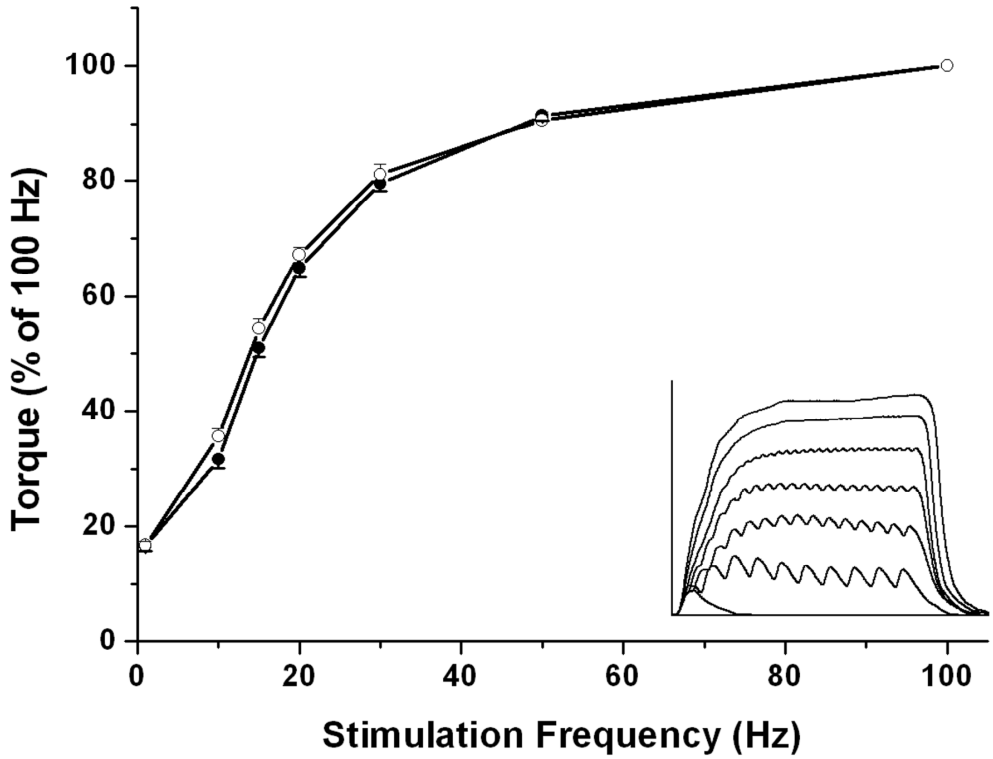

Figure 2.1. The torque-frequency curve of the quadriceps muscle determined by electrical stimulation in men (●) and women (○). Values are expressed as a percentage of the torque generated by 100 Hz stimulation. Error bar represent SEM. Insert shows a typical series of torque records when stimulating at 1, 10, 15, 20, 30 50 and 100 Hz.

women respectively. Maximal voluntary torque and ACSA were lower in women ($P<0.001$), but there were no significant differences between men and women in their ability to voluntarily activate their quadriceps muscles (Table 2.1).

Torque-frequency relationship and maximum relaxation rate

Individual torque–frequency curves were normalised by expressing the data relative to peak (100 Hz) torque (Figure 2.1). Women generated marginally more torque at low frequencies of stimulation, but this was not significant. Only the percentage torque at 10 Hz was 4 % higher in women compared with men but this did not reach statistical significance ($P=0.063$; Figure 2.1), while at 15 Hz and 20 Hz no differences were observed ($P=0.15$ at 15 Hz and $P=0.26$ at 20 Hz). Contractile speed, as assessed by the rate of relaxation (MRR_{30}) from a 30 Hz tetanus, of the female muscles was appreciably slower than that of the men ($P=0.002$; Table 2.1).

Fatigue tests

Torque

The development of fatigue, as indicated by the progressive decline in torque during the two minutes of the fatigue tests, is shown in Figure 2. In the standard fatigue test (Figure 2.2A) torque declined significantly more in men (by 37.7 ± 10.7 %) than in women (by 29.9 ± 10.0 %, $P<0.01$).

Decreasing the recovery time between the tetani from 1 s to 0.5 s (Figure 2.2B) increased the rate of fatigue and differences between men and women became apparent earlier than during the standard fatigue test; i.e. after 37.5 s as opposed to 78 s into the standard fatigue test. Using this protocol, the differences in torque decline between men and women also became more marked (55.7 ± 8.1 *vs.* 42.8 ± 13.4 % decline; men *vs.* women, $P<0.01$).

The standard fatigue test carried out under ischaemic conditions (Figure 2.2C) visually showed a slightly different time course than in either of the other two protocols.

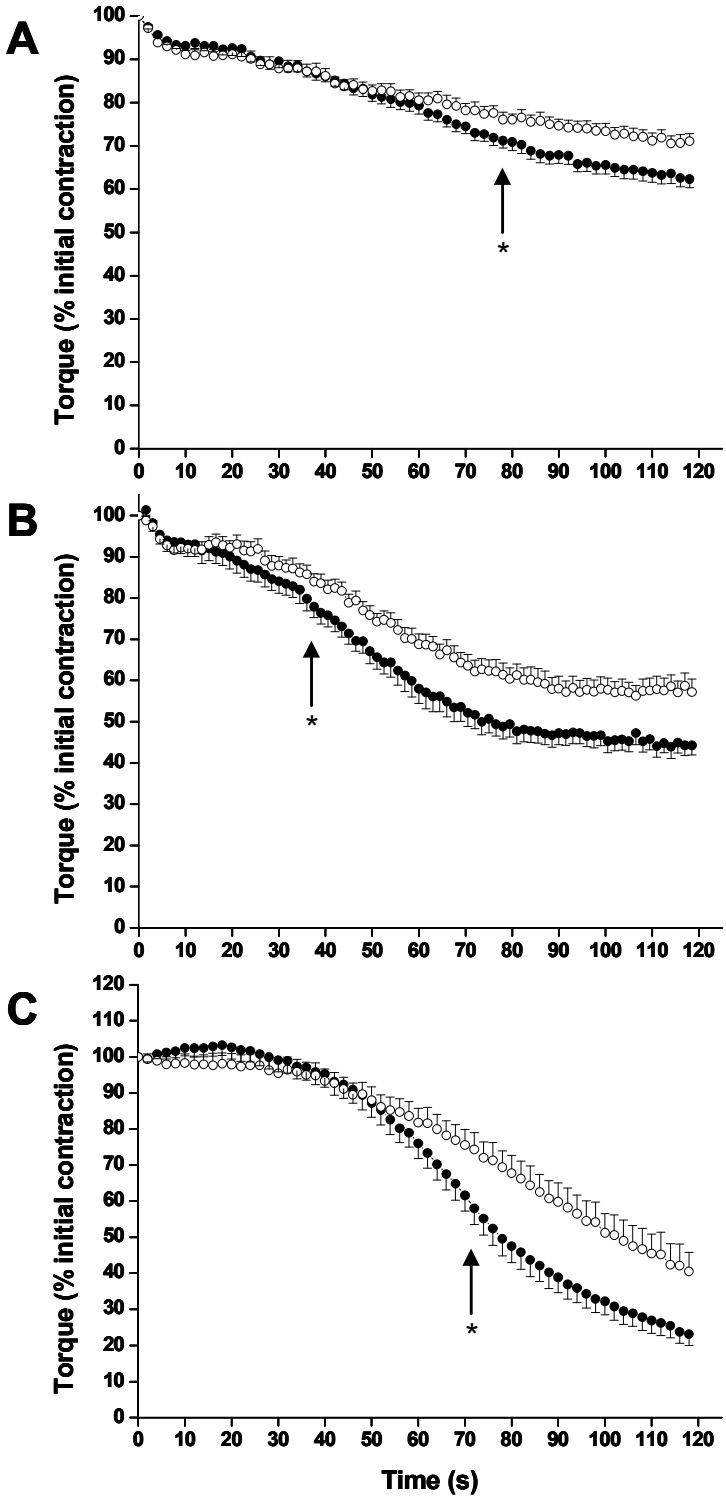

Figure 2.2. Torque during the fatigue protocols in men (●) and women (○). The decline in torque is expressed as percentage of the initial torque during the fatigue protocol for (**A**) 1 s on, 1 s off (29 men and 35 women), (**B**) 1 s on, 0.5 s off (12 men and 18 women) and (**C**) 1 s on, 1 s off, with circulatory occlusion (12 men and 10 women). Data are plotted every 2 s during the complete stimulation protocol for both sexes. Error bars represent SEM. Torque declined significantly more in men than women (all tests $P<0.01$). Arrows indicate the first contraction with a significant difference between the sexes (after 78 s, 37.5 s and 72 s for **A**, **B** and **C** respectively).

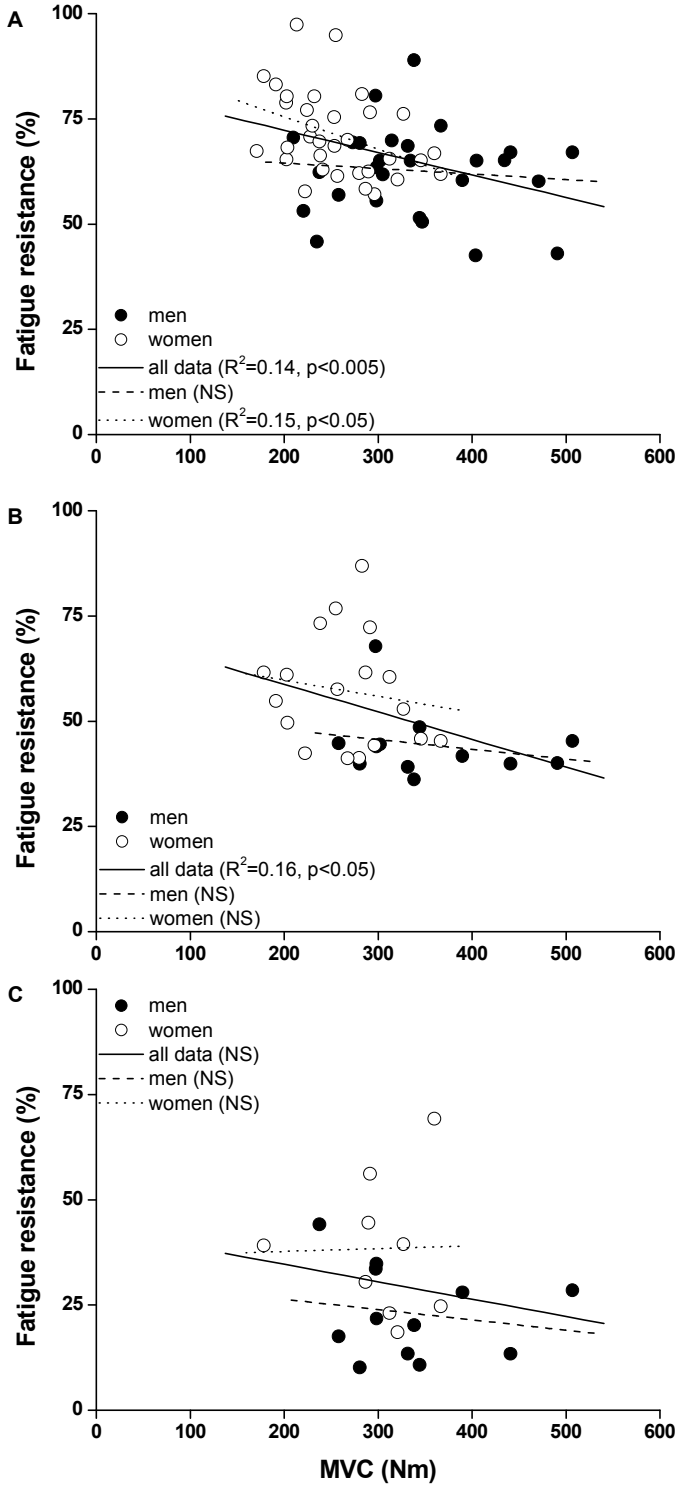

Figure 2.3. Relationship between the torque during a maximal voluntary contraction (MVC) and fatigue resistance (remaining torque as a percentage of initial torque, in %) for men (●) and women (○). Note that while pooling all the data, significant correlations were observed in the non-occluded fatigue test (**A**: standard (29 men and 35 women) and **B**: 0.5 s off (12 men and 18 women)), while this relation did not occur within sexes. In the occluded standard fatigue test (**C,** 12 men and 10 women), no significant correlations were observed.

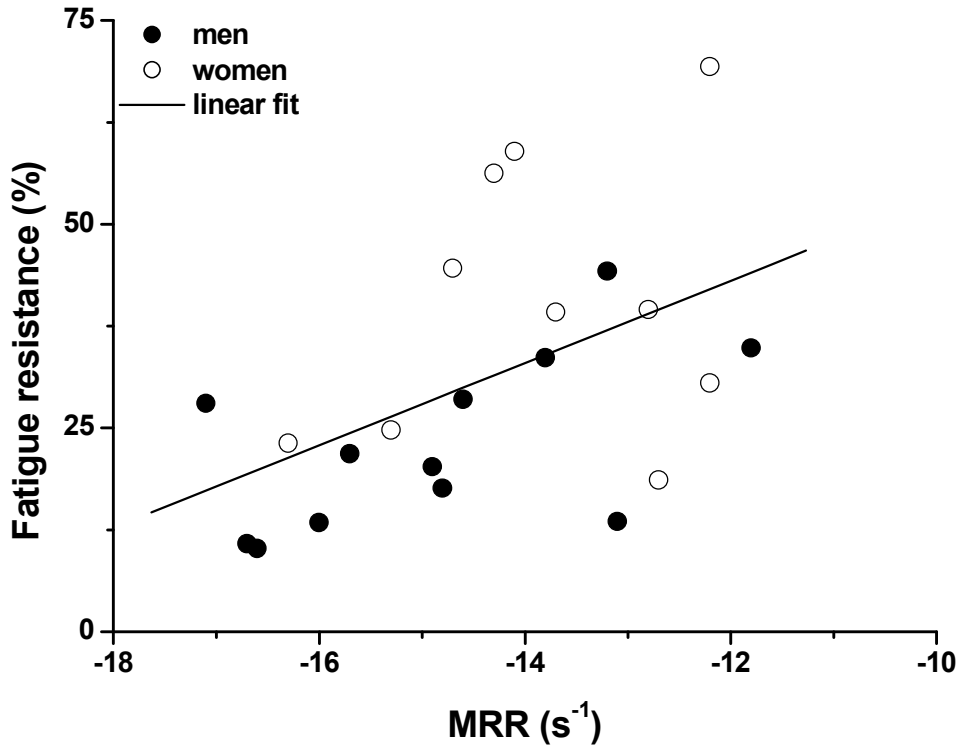

Figure 2.4. Significant relationship between the maximal rate of relaxation (MRR) and fatigue resistance (remaining torque as a percentage of initial torque, in %) for men (●) and women (○) during the occluded standard fatigue test (R^2=0.40, P=0.002; 12 men and 10 women).

Significant differences between men and women became apparent after 72 s and the difference in loss of torque between men and women (76.9 ± 10.8 *vs.* 59.5 ± 16.9 %; men *vs.* women, P<0.01) was greater than in either of the fatigue tests with intact circulation. Our hypothesis that sex-related differences would be abolished under ischaemic conditions had to be rejected, as we observed that in the standard and ischaemic fatigue test, women fatigued about a third less than men.

The subjects who had larger and stronger muscles, in general the men, also tended to fatigue most rapidly. This was reflected by the significant correlation between MVC and fatigue resistance (i.e. remaining torque as a percentage of the initial value) for the pooled data of the standard fatigue test (Figure 2.3A). However, this correlation does not necessarily indicate a causal relationship. Indeed, for the standard protocol (Figure 2.3A) there was a weak relationship between strength and fatigability within

the women only, but not in the men. In the second protocol, the recovery interval was shortened (Figure 2.3B) and hence the duration of occlusion of the circulation is longer. The weak correlation between MVC and fatigue resistance in the women even disappeared, although for the combined data there remained a weak relationship. To entirely remove the possible role of differences between sexes in occlusion of the circulation during the fatigue tests an ischaemic fatigue protocol was performed (Figure 2.3C). In this case the absolute torque would not affect blood flow as it is already minimised. As expected no significant relationships between strength and fatigue, either for each sex separately or for the pooled data were found.

A significant relationship existed between the MRR of the unfatigued muscle and the decline in torque in the standard fatigue test (R^2=0.12; P=0.005), the second fatigue protocol (R^2=0.26; P<0.001) and the standard fatigue test under ischaemic conditions (R^2=0.40; P=0.02; Figure 2.4). Similar results were obtained from the men and women separately. Moreover, the correlations did not change after controlling for MVC.

Discussion

Our results add weight to those reports suggesting women are more fatigue resistant than men. The new finding is that the difference between men and women is evident during electrically stimulated contractions, which eliminates any possible problems associated with activation. Moreover, it persists during a fatigue protocol that largely eliminated oxidative phosphorylation, indicating that the key difference does not involve questions of blood flow or the oxidative capacity of the muscles. Our results show that the difference in fatigability between men and women is a consequence of higher energy utilisation in men and could be related to the differences in contractile speed that we have observed.

Central and peripheral fatigue

Maintaining continuous, or even intermittent, muscular contractions is a demanding task that requires concentration and motivation. It is conceivable that one sex may be slightly better at these tasks accounting for the differences that have been reported with fatigue protocols involving voluntary activation. However, Hunter *et al.* (2006a), using a superimposed twitch technique, have shown that the decline in activation during a series of voluntary contractions was similar in men and women.

We have taken this one step further by both fatiguing and testing the muscle using electrical stimulation, a situation where voluntary activation plays no role. Consequently, the observed difference in fatigability clearly has a peripheral cause as opposed to an origin in the central nervous system. In addition this method also circumvents possible bias related to motor unit rotation that may occur during voluntary contractions, all factors that have been suggested to play a role in the sex-related differences in muscle fatigue (Hicks *et al.*, 2001; Clark *et al.*, 2003; Hunter *et al.*, 2004b; Clark *et al.*, 2005). The question therefore is what aspect of peripheral muscle physiology or metabolism can account for the differences in fatigability between men and women?

Transmission failure

During repetitive electrical stimulation there is the possibility that transmission at the neuromuscular junction may be impaired, possibly as a result of acetyl choline depletion, or failure of action potential propagation along the sarcolemma or T-tubule membranes. The protocol we have used was designed to minimise the likelihood of such transmission failure by keeping the stimulation frequency relatively low (30 Hz) and allowing repeated recovery intervals since transmission failure recovers rapidly when the contraction stops (Jones, 1996). It would appear, therefore, that differences between men and women are unlikely to be due to differences in transmission across the neuromuscular junction or along the sarcolemma and T-tubular membranes.

Oxidative metabolism

The rate of fatigue during the standard protocol we have used will be a balance between the depletion of energy stores during activity and the extent of recovery metabolism. In turn, the extent of aerobic recovery will depend on the oxidative capacity of the fibres, the degree to which blood flow is occluded during the contraction, and the duration of the recovery period between contractions. Given a long enough interval there will be complete recovery, while shortening the interval will increase the stress on the oxidative processes to fully replenish the energy stores. When, in the second protocol, there was only 0.5 s between the contractions and relatively less blood flow and thus aerobic recovery, the rate of fatigue was increased but, interestingly, the women fatigued a third less compared to the men, similar to the

results we found in the standard fatigue test. This could be reflection of a lower capacity of aerobic energy regeneration in men than women and/or a higher rate of energy utilisation in men than women.

Our male subjects were, on average, stronger and had larger quadriceps muscles and it is possible, therefore, that the blood supply to the working muscles during the contraction phase was impeded to a greater extent in the larger and stronger male than female muscles. The data shown in Figure 2.3A, suggest that there may be a small component of fatigue in the female participants which could be explained in this way but there is not a simple relationship between force and blood flow. Considerable intramuscular pressures are developed in even comparatively small rabbit muscles (Degens *et al.*, 1998) and it has been shown that the oxygenation of the muscle depends on the relative, rather than the absolute, torque the muscle generates during a contraction (De Ruiter *et al.*, 2007). However, the most persuasive argument that differences in fatigability between men and women has nothing to do with blood flow or oxidative capacity of the muscle is our observation that the differences between men and women were still evident when the blood flow was occluded. This clearly shows that there are differences between male and female muscle that must reside in the rate at which energy is used by the muscle fibres rather than the oxidative recovery rates. We conclude that the observation that men, with stronger muscles, also fatigue more rapidly, is coincidental and not causal, there being some other reason for the faster rate of fatigue.

Our results contrast with the findings presented by Russ and Kent-Braun (2003), who reported that sex differences in muscle fatigue were absent under ischaemic conditions and concluded that blood flow and/or substrate utilisation differ between men and women. The difference between the present study and their results may be due to the study design. Problems of voluntary activation become very important during sustained ischaemic contractions and this may well have obscured any differences between male and female subjects, and it is possible that there may be differences between the calf muscles used by Russ and Kent-Braun (2003) and the quadriceps muscles investigated here.

Possible differences in muscle fibre *characteristics between men and women*

Compared to type II fibres, type I fibres have a slower speed of contraction and consequently a slower rate of energy utilisation (Stienen *et al.*, 1996; Hamada *et al.*,

2003) and, characteristically, fatigue at different rates and this can be seen in the fatigue characteristics of human muscle (Gordon *et al.*, 1990; Hamada *et al.*, 2003). The female participants had muscles that were, on average, slower than those of the males. There was a small, although not significant, difference in the torque-frequency relationship (Figure 2.1) and the MRR was significantly slower (Table 2.1). The relationship between MRR and rate of fatigue (Figure 2.4) suggests that the fatigability of the muscle is related to the contractile speed; the slower the muscle the more fatigue resistant (Gordon *et al.*, 1990; Hamada *et al.*, 2003). The logic of this argument is that female muscle should contain more type I slow fibres than male muscle. However, there is controversy as to whether male and female muscle differ in their fibre type composition but the available evidence tends to show a (5 %) larger percentage of type I fibres in pre-menopausal women than men (Nygaard, 1981; Simoneau *et al.*, 1985; Simoneau & Bouchard, 1989; Mannion *et al.*, 1997; Staron *et al.*, 2000; Jaworowski *et al.*, 2002; Roepstorff *et al.*, 2006; Yu *et al.*, 2007). Interestingly, when extrapolating the data from Hamada *et al.* (2003), a 5 % change in the proportion of type I fibres would result in a ~7.5 % change in fatigue resistance during a non occluded, volitional fatigue test.

Conclusion

In conclusion, we can confirm that fatigue resistance during a series of intermittent contractions is lower in men compared to women when matched for age and physical activity. As the differences were observed using electrical stimulation, we can conclude that this higher fatigue resistance has a peripheral origin. It is unlikely that differences in blood supply and/or oxidative phosphorylation underlie this difference, as fatigue resistance was also higher in muscles from women than men when the circulation was occluded. Intrinsic sex-related differences in skeletal muscle properties, such as contractile speed and rate of energy utilisation probably play a key role in the sex related differences in fatigue.

Acknowledgements

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CHAPTER 3

MUSCLE FATIGUE RESISTANCE DURING STIMULATED CONTRACTIONS IS REDUCED IN YOUNG MALE SMOKERS

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Abstract

To determine whether muscle function is compromised in healthy smokers in comparison with activity-matched non-smokers, nine male smokers (aged 22.2 ± 2.5 yr: mean ± SD) with a smoking history of 2.5 ± 3.1 pack years, and ten male control participants (25.4 ± 2.9 yr) matched for physical activity level participated in this study. Knee extensor strength was measured using Isometric maximal voluntary contractions. Voluntary activation of the quadriceps and co-activation of the biceps femoris were determined using interpolated twitches and surface electromyography, respectively. The frequency-torque relationship and fatigue resistance were assessed with electrically evoked contractions. A fatigue index was determined as the ratio of final torque to initial torque during series of isometric contractions (1s on 1 s off, 30 Hz, 2 min). Quadriceps anatomical cross sectional area was measured with MRI at 50 % of femur length. Maximal voluntary contraction torque, quadriceps anatomical cross sectional area, knee extensor torque/quadriceps cross sectional area, activation, co-activation and force-frequency relationship were similar, whereas the fatigue index was 17 % lower in smokers than non-smokers. In young men smoking does not significantly affect quadriceps muscle cross sectional area and contractile properties, but does reduce fatigue resistance of the quadriceps muscle, which was not attributable to differences in physical activity.

Introduction

Smoking is a recognised risk factor for cardiovascular disease, lung cancer and chronic obstructive pulmonary disease (COPD). Many patients with COPD and chronic heart failure suffer from muscle dysfunction, which is not directly related to changes in lung or heart function (Gosker *et al.*, 2000). Besides muscle wasting, which results in weakness (Franssen *et al.*, 2005), there is a reduction in the oxidative capacity (Gosker *et al.*, 2000) and a shift towards faster fibre types (Satta *et al.*, 1997) which may explain the reduced muscle endurance in COPD patients (Van 't Hul *et al.*, 2004).

In addition to damaging the lungs, smoking may also affect skeletal muscle (Agusti *et al.*, 2003; Gan *et al.*, 2004; Gan *et al.*, 2005). Smoking has been reported to cause a decline in muscle strength (Örlander *et al.*, 1979; Al-Obaidi *et al.*, 2004). As muscle cross-sectional area is the major determinant of strength (Maughan *et al.*, 1983) it is likely that the lower muscle strength in smokers is attributable to a smaller muscle mass. However, where differences in muscle strength have been reported between smokers and non-smokers, muscle size has not been measured. Furthermore, a shift from high-oxidative type I (slow-twitch) fibres towards a higher percentage of low-oxidative type II (fast-twitch) fibres and a reduction in fibrillar mitochondrial volume fraction occurs (Örlander *et al.*, 1979); changes that are likely to result in a reduction of fatigue resistance (Degens & Veerkamp, 1994). It has been suggested that the differences in skeletal muscle between smokers and non-smokers is at least partly due to a lower physical activity level of smokers (Örlander *et al.*, 1979). It is important to minimize this bias when studying the effects of smoking on skeletal muscle. Indeed, disuse induces the same changes in skeletal muscle. Therefore, it is important to take into account the physical activity level to separate the effects of disuse from those of smoking *per se*.

Thus, the aim of the present investigation was to determine whether i) smokers are weaker than non-smokers, ii) any weakness is the result of neural and/or muscular factors, iii) there is any difference in contractile properties between smokers and non-smokers and iv) fatigue resistance is altered in smokers compared to non-smokers. To exclude the role of disuse, control male participants were activity-matched with

healthy smokers. To circumvent possible differences in motivation between smokers and non-smokers, electrically evoked muscle contractions were used to test muscle function. Considering the role of smoking in the development of COPD and its potential effects on skeletal muscle, the present study may reveal changes in skeletal muscle function of young healthy smokers (i.e., free from any pre-existing medical condition) before any clinical signs of COPD develop.

Methods

Participants

Table 3.1. Participant characteristics

	Smokers (n=9)	Non-smokers (n=10)
Age (years)	22.2 ± 2.5	25.4 ± 2.9
Mass (kg)	79.9 ± 14.0	76.1 ± 8.7
Height (cm)	180.2 ± 6.5	179.9 ± 6.4
BMI (kg m^{-2})	26.6 ± 2.3	23.5 ± 1.5
FVC (L)	4.4 ± 0.8	5.0 ± 0.6
FEV_1%pred	91.3 ± 13.7	104.7 ± 9.9
Physical activity score	8.3 ± 1.6	8.7 ± 1.5
Smoking history (pack years)	2.5 ± 3.1	-

BMI, body mass index; VC, vital capacity; FEV_1%pred, the percentage of the age and height adjusted predicted FEV_1.

Nine male smokers and ten male non-smokers (Table 3.1) participated in the study. Smokers and non-smokers were matched for age and physical activity level. Written informed consent was obtained from all participants and all procedures were approved by the local ethics committee of Manchester Metropolitan University. Potential participants who suffered from respiratory, cardiovascular or neuromuscular diseases or had any lower limb injury were excluded from the study. All participants abstained from smoking and caffeine ingestion for two hours prior to testing. Physical activity (PA) levels were assessed using a previously validated questionnaire (Baecke *et al.*, 1982); where <6 indicates a sedentary lifestyle and >9 a high physical

activity level. All participants were relatively inactive with a mean score of around 8-9 for each group (Table 3.1). Smoking history was assessed by questionnaire and smoking volume was determined by pack-years, which is defined as the number of cigarette packs smoked per day, multiplied by the number of years smoking. The vital capacity (VC) and forced expiratory volume in 1 second (FEV_1) was assessed with spirometry (Vitalograph Ltd. Bucks, UK) and used to determine the percentage of the age- and height-adjusted predicted value (FEV_1%pred; Crapo *et al.*, 1981).

Experimental set-up

Participants were familiarised with the testing procedures on a separate session prior to data collection. All knee extension torque measurements were performed on the right leg with a Cybex norm dynamometer (Ronkonkoma, New York, USA). Participants were seated with the hip joint at 90° flexion with the hip and shoulders strapped to prevent any extraneous movement. Before the experiment, participants warmed-up by performing 5 submaximal isokinetic contractions. Torque was displayed on a computer screen, interfaced with an acquisition system (Acknowledge, Biopac Systems, Santa Barbara, CA, USA) used for analogue-to-digital conversion. The sampling frequency was 2000 Hz. Each torque signal was filtered with a low-pass fourth order Butterworth filter with a 30 Hz cut-off frequency.

Isometric maximal voluntary contractions (MVC) torque.

The optimal angle for torque generation was 80° (full knee extension=0°) in all but two participants (data not shown). Therefore, to reduce the influence of joint angle on twitch characteristics all subsequent measurements were performed at a knee joint angle of 80°. MVCs were maintained for 4 seconds (sufficient to reach a plateau) with 2 min of rest in between each contraction to prevent development of fatigue. To maximize performance, visual feedback of the torque signal and verbal encouragement was given to all participants. The highest torque reached during a contraction was recorded, and the highest value of the two contractions was given as the MVC.

Voluntary activation

Voluntary activation levels were determined with the interpolated twitch technique as described previously (Allen *et al.*, 1995). Briefly, the muscle was stimulated percutaneously, with the anode (76 mm x 127 mm, Versastim, Conmed Corp., N.Y., USA), placed over the proximal region of the quadriceps and the cathode over the distal third of the femur length. To assess the supra-maximal current, single pulses (pulse width 50 μs; DSV Digitimer Stimulator, Digitimer Ltd., Herts, UK) were administered at 30 s intervals with increases in current of 50-100 mA, until no further increase in torque was observed. For the interpolated twitch technique, a first doublet (pulse width of 50 μs, 100 Hz) was applied with the participant in a relaxed state and a second during the plateau phase of the MVC. The ratio of interpolated and resting doublets was used to provide an index of activation (Allen *et al.*, 1995) as follows:

$$\text{Voluntary activation (\%)} = 100 \times \left(1 - \left(\frac{\text{superimposed doublet torque}}{\text{resting doublet torque}}\right)\right)$$

where the superimposed doublet torque is the additional torque during the MVC caused by the doublet.

Antagonist co-activation

To determine whether antagonist co-contraction affected the measured torque during a knee extensor MVC, the electromyographic (EMG) activity of the biceps femoris was recorded as described previously (Kellis & Baltzopoulos, 1997). Two 10-mm, pre-gelled, Ag-AgCl percutaneous unipolar electrodes (Medicotest, Olstykke, Denmark) were placed along the mid sagittal line of the biceps femoris muscle to reduce the level of cross talk. To ensure that EMG recordings were made beyond the motor point of the biceps femoris, electrodes were positioned at a third of total femur length (Zipp, 1982), with the reference electrodes placed over the lateral epicondyle of the femur. The raw EMG activity was acquired with a sampling frequency of 2000 Hz and processed with a multi channel analogue-digital converter (Biopac Systems Inc., CA, USA). The raw EMG signal was filtered with low and high-band pass filters set at 500 Hz and 10 Hz respectively, and amplified with a gain of 2000. The level of co-activation of the biceps femoris was assessed using the root mean square

(RMS) of the raw EMG signal which was integrated over 1 s about the peak MVC torque during knee extension and was expressed as the percentage of activity recorded from the biceps femoris during maximal knee flexion (Klein *et al.*, 2001).

Contractile properties

The frequency-torque relationship and fatigue resistance were determined with electrically evoked contractions essentially as described previously (Degens *et al.*, 2005). Briefly, the intensity of stimulation throughout the test was such that about 30 % of MVC toque was developed during a 100 Hz contraction.

Five minutes after the last MVC, the quadriceps muscle was stimulated with 1, 10, 15, 20, 30, 50 and 100-Hz trains for 1 s, separated by 1 min, to assess the frequency-torque relationship. Frequencies were delivered in a random order. From the 100 Hz tetanus of the frequency-torque relationship, the contraction time was measured as the time from activation until 90 % of the maximal torque was reached, and the relaxation time as the time for the torque to decline to 50 % of maximal torque after cessation of stimulation (Degens *et al.*, 2005).

Fatigue test

Five minutes after the contractions for the frequency-torque relationship the resistance to fatigue was assessed by stimulating the quadriceps muscle with 30 Hz trains, 1 s on 1 s off, for 2 minutes (Degens *et al.*, 2005). Fatigue index (FI) was expressed as the torque at the end of the test divided by that at the start of the test.

Anatomical cross-sectional area

Anatomical cross-sectional area (ACSA) of the quadriceps was measured with magnetic resonance imaging (MRI), using a fixed 0.2 T MRI scanner (E-Scan, ESAOTE Biomedica, Genova, Italy), at 50% of femur length. Scans were obtained with a T1 weighted, high resolution, gradient echo profile, with the following scanning parameters: echo time: 0.016 s; repetition time: 0.1 s; field of view: 0.33 x 0.254 m and a slice thickness of 0.005 m. Subjects were supine for 15 min prior to the scan to make sure any fluid shifts had stabilised. To account for differences in muscle cross-sectional area between the two groups, maximal knee extensor torque was scaled with quadriceps ACSA (Maughan *et al.*, 1983) and expressed as MVC/ACSA (Nm cm^{-2}).

Statistical analysis

Data are reported as means (SD). To determine the effects of smoking an unpaired two-tailed Student *t*-test was performed. None of the parameters violated the assumptions of the *t*-test except for physical activity scores which were compared using a non-parametric Mann-Whitney test. Differences were considered significant at $P<0.05$.

Results

Subject characteristics

Table 3.1 shows the subject characteristics. Body mass, stature and body mass index (BMI) did not differ between smokers and non-smokers (Table 3.1). The smokers and non-smokers were successfully matched for physical activity as the PA scores did not differ significantly (Table 3.1). Smoking history ranged from 0.4 - 11.1 pack years, with a median of 1.7 years (Table 3.1). The non-smokers had never smoked. VC and FEV_1%pred did not differ significantly between smokers and non-smokers (Table 3.1).

Table 3.2. Quadriceps function and contractile characteristics from male smokers and non-smokers

	Smokers (n=9)	Non-smokers (n=10)
MVC (Nm)	335 ± 73	351 ± 72
ACSA (cm^2)	74.9 ± 11.6	78.9 ± 6.7
MVC/ACSA (Nm cm^{-2})	4.5 ± 0.5	4.5 ± 0.9
Activation (%)	92 ± 3	93.3 ± 8.6
Co-activation (%)	27 ± 17	18 ± 10
Contraction time (ms)	334 ± 125	293 ± 67
Half relaxation time (ms)	112 ± 6	117 ± 8

MVC, maximal voluntary isometric knee extension torque; ACSA, anatomical cross-sectional area; MVC/ACSA, MVC torque relative to ACSA; for further details see the *Methods* section.

Strength and muscle size

Knee extensor MVC torque was not significantly different between smokers and non-smokers (Table 3.2). This and the similar ACSA were reflected in the absence of a significant difference in the MVC/ACSA between smokers and non-smokers (Table 3.2). Also, the level of voluntary activation and co-activation of the antagonist muscles during an MVC were not significantly different between smokers and non-smokers (Table 3.2).

Contractile properties

The contraction times of the 100 Hz tetani were not significantly different between the smokers and non-smokers (Table 3.2). Similarly, half relaxation time was comparable in the two groups. In line with this the frequency-torque relationship of the quadriceps of smokers and non-smokers showed no difference (Figure 3.1).

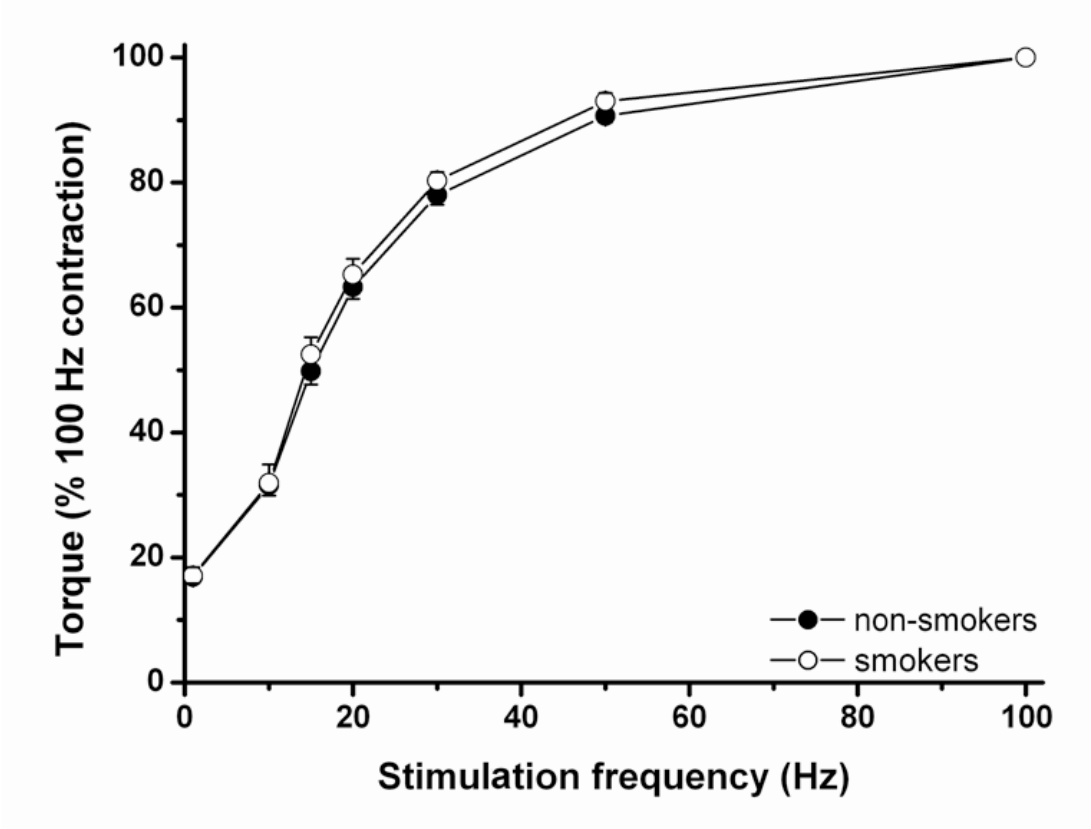

Figure 3.1. The frequency-torque relationship in healthy young male smokers (○, n=9) and non-smokers (●, n=10).

Fatigue index

In figure 3.2 it can be seen that the quadriceps muscles of the smokers were more susceptible to fatigue than those of the non-smokers ($P<0.05$); after two minutes of 30 Hz stimulation the FI was 54 ± 8 *vs.* 62 ± 9 % in smokers and non-smokers, respectively. The variation in FI among the smokers did not correlate with either smoking volume in terms of pack years ($R^2=0.09$, $P>0.05$) or FEV_1%pred ($R^2=0.28$, $P>0.05$).

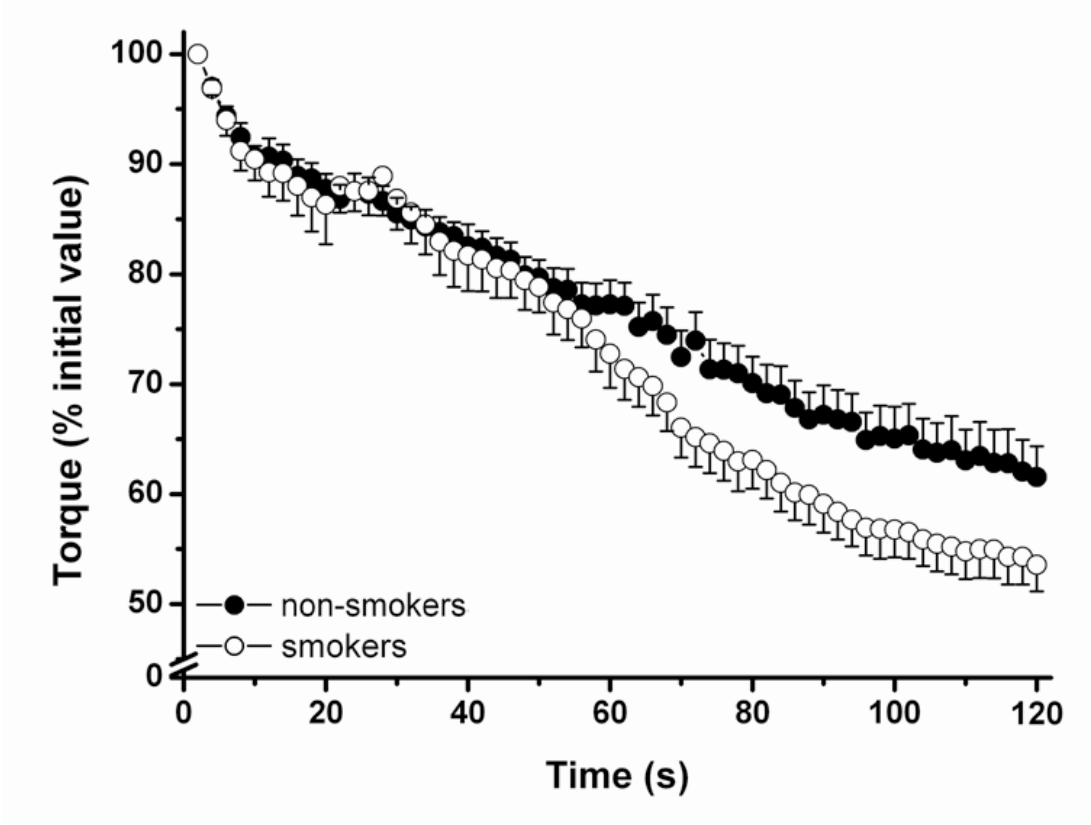

Figure 3.2. Fatigue resistance of the quadriceps muscle in healthy young male smokers (○, n=9) and non-smokers (●, n=10). The torque, elicited by electrical stimulation (1 s on 1 s off, 30 Hz) of the quadriceps muscle, is presented as relative to the torque generated during the first contraction. The torque decline was larger in smokers than non-smokers ($P<0.05$).

Discussion

The main finding of the present study was a decrease in fatigue resistance in young male smokers compared with a group of physical activity-matched non-smokers, while strength and other contractile properties did not differ between the groups. Based on the present results the decrease in fatigue resistance cannot be explained by

disuse, or restricted airflow. The electrically stimulated fatigue test eliminates the possibility that differences in fatigability are due to differences in motivation and it is probable that the cause of the lower fatigue resistance in smokers is located within the muscle, at or beyond the level of the neuromuscular junction.

Strength

We observed no differences in knee extension strength between smokers and non-smokers. Two other studies, however, have reported a decline in maximal voluntary strength in smokers (Örlander *et al.*, 1979; Al-Obaidi *et al.*, 2004). Such a decline can, at least theoretically, be a consequence of a smaller muscle mass, a reduced ability to voluntarily activate the muscle and/or increased activation of antagonistic muscles during a maximal contraction. These factors could be secondary to lower levels of activity in the smokers in these previous studies. Indeed, it has been suggested that a lower level of physical activity underlies the decline in strength previously observed in smokers (Örlander *et al.*, 1979). In the present study, smokers and non-smokers were matched for physical activity level and, similarly to Larsson *et al.* (1984), we did not observe a difference in strength between smokers and non-smokers. Thus, the decrease in strength observed in previous studies is likely to be explicable by a reduced activity level in smokers, rather than an effect of smoking *per se* on maximal knee extensor strength or other determinants of strength (activation, co-activation and ACSA).

Fatigue

Although strength was not affected by smoking, the fatigue resistance of the quadriceps muscle was significantly reduced. Others, however, have not observed a change in the fatigue resistance during maximal voluntary contractions in smokers (Örlander *et al.*, 1979; Larsson, 1984). This discrepancy is difficult to explain but may, in part, be related to the type of fatigue tests performed. Previous studies have used a series of fast dynamic contractions where the recovery interval between contractions was probably around half a second, compared to the one second interval in the present study. Dynamic contractions are more energetically demanding than the isometric contractions we have used. Consequently, previous work may have been testing predominantly anaerobic function whilst our protocol emphasises more

the aerobic capacity of the muscle and the ability to recover between successive contractions.

The acute neural stimulation effect of nicotine in cigarette smoke is well established (Cryer *et al.*, 1976) and Mündel and Jones (2006) have demonstrated that nicotine has a beneficial effect on cycling endurance. It might be hypothesised that the stimulant effect of nicotine during voluntary fatigue tests may counteract some peripheral mechanism which contributed to the greater levels of fatigue in the smokers in the present study.

Smoking is the primary cause of COPD, which is characterised by a decline in FEV_1, and it has been reported that smoking itself causes an increase in pulmonary airway resistance (Nadel & Comroe, 1961). However, we did not observe a significantly lower FEV_1%pred in smokers than non-smokers, or any correlation between fatigue resistance and FEV_1%pred. Therefore, it is unlikely that lung function and the ability to oxygenate the blood play any role in the lower fatigue resistance of smokers. Indeed, as the fatigue test was performed on one leg, with activation of only about 30% of the quadriceps, blood oxygenation was probably not the predominant limiting factor.

Lower levels of habitual physical activity level might lead to reduced oxidative capacity, but in the present investigation the similar activity levels suggest that it is cigarette smoking itself which is responsible for the observed reduction in fatigue resistance.

A number of mechanisms (both acute and chronic) may be responsible for the observed decline in fatigue resistance, these include alterations in muscle fibre type composition and/or a decreased oxidative capacity (Örlander *et al.*, 1979; Larsson, 1984). Although the frequency-torque relationship and contraction and relaxation times are not unequivocal indicators for changes in fibre type composition, the absence of significant alterations in these parameters indicates no major changes in fibre type composition.

Mitochondrial enzyme activities (citrate synthase and 3-hydroxyacyl-CoA dehydrogenase) have been reported to be decreased in the muscles of smokers (Örlander *et al.*, 1979). Interestingly, healthy ex-smokers did not show a decline in

activities of enzymes of oxidative metabolism (Larsson, 1984) and COPD patients who stopped smoking exhibited normal fatigue resistance (Degens *et al.*, 2005). This suggests that the smoking-induced alterations in the activity of oxidative enzymes and muscle fatigue resistance might be reversible. Furthermore, the fact that we found no relationship between pack years and fatigue resistance suggests an acute effect of smoking. Cyanide and carbon monoxide (CO) in smoke are possible candidates for acutely affecting fatigue resistance. Cyanide and CO inhibit cytochrome *c* oxidase (complex IV in the mitochondrial chain), causing an overall decline in mitochondrial function and oxidative capacity (Alonso *et al.*, 2003), and thus potentially a reduced fatigue resistance of the muscle.

Besides acting on cytochrome *c* oxidase, CO in cigarette smoke increases carboxyhemoglobin (COHb) levels (Puente-Maestu *et al.*, 1998), which reduces the O_2 carrying capacity of the blood. However, a reduced O_2 carrying capacity of the blood induced by breathing hypoxic air had no effect on fatigue resistance during the same test as employed in the present study (Degens *et al.*, 2006). COHb also hampers the release of O_2 as reflected by the left-ward shift of the O_2-Hb binding curve. Furthermore, the facilitated diffusion of O_2 by myoglobin within the muscle may be impaired by the formation of carboxymyoglobin (Wittenberg & Wittenberg, 1987). It has been reported that raised levels of COHb reduce O_2 extraction during stimulated contractions with *in situ* canine gastrocnemius, which was accompanied by a reduction in muscle tension (King *et al.*, 1987).

Besides changes in the muscle itself, another possibility for the earlier onset of fatigue in smokers might be related to neurotransmission failure. Using direct muscle stimulation superimposed on nerve stimulation, it has been shown that neurotransmission failure contributes to an earlier onset of fatigue during hypoxia (Zhu *et al.*, 2006). With the present stimulation protocol we are unable to state whether neural transmission failure contributed to the earlier onset of fatigue in smokers.

We assume that the more rapid fatigue, whatever the cause, is a consequence of changes occurring as a result of smoking. However, we cannot exclude the possibility that there may be some genetic component that, for instance, influences the likelihood of smoking and, at the same time, influences fatigue properties of the muscle.

The relationship between the changes in fatigue resistance we report here and the muscle dysfunction frequently seen in COPD patients is unclear. Given that fatigue resistance is not affected in COPD patients who stopped smoking (Degens *et al.*, 2005) and the fact that we saw no association between pack years smoked and FI, it is likely that the reduced fatigue resistance in the present smokers is acute in nature. If it is an acute response, the underlying factors of this fatigue are most likely changes in the oxidative capacity of the muscle brought about, for instance, by a reversible effect of cyanide and/or CO on mitochondrial enzyme function and tissue oxygenation. Indeed, 28 days of smoking cessation has been shown to result in a return to normal mitochondrial respiratory chain function (Cardellach *et al.*, 2003). Therefore, future studies may be directed towards the acute effects of CO, smoking and smoking cessation on skeletal muscle function, oxygenation and fatigue resistance.

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CHAPTER 4

SKELETAL MUSCLE PROPERTIES AND FATIGUE RESISTANCE IN RELATION TO SMOKING HISTORY

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Abstract

Although smoking-related diseases, such as chronic obstructive pulmonary disease (COPD), are often accompanied by increased peripheral muscle fatigability, the extent to which this is a feature of the disease or a direct effect of smoking *per se* is not known. Skeletal muscle function was investigated in terms of maximal voluntary isometric torque, activation, contractile properties and fatigability, using electrically evoked contractions of the quadriceps muscle of 40 smokers (19 men and 21 women; mean ± SD) cigarette pack years: 9.9 ± 10.7) and age- and physical activity level matched non-smokers (22 men and 23 women). Maximal strength and isometric contractile speed did not differ significantly between smokers and non-smokers. Muscle fatigue (measured as torque decline during a series of repetitive contractions) was greater in smokers (P=0.014), but did not correlate with cigarette pack years (r=0.094, P=0.615), cigarettes smoked per day (r=-0.092, P=0.628), respiratory function (%FEV_{1pred}) (r=-0.187, P=0.416), or physical activity level (r=-0.029, P=0.877). While muscle mass and contractile properties are similar in smokers and non-smokers, smokers do suffer from greater peripheral muscle fatigue. The observation that the cigarette smoking history did not correlate with fatigability suggests that the effect is either acute and/or reaches a ceiling, rather than being cumulative. An acute and reversible effect of smoking could be caused by carbon monoxide and/or other substances in smoke hampering oxygen delivery and mitochondrial function.

Introduction

Smoking is a recognised risk factor for many chronic diseases, such as chronic obstructive pulmonary disease (COPD). Patients with COPD often complain of exercise intolerance and fatigue, which can be attributed to deterioration in lung function and systemic effects such as muscle atrophy and a slow-to-fast transition in fibre type composition (Chapter 1). However, also otherwise non-symptomatic smokers often complain of an increased sensation of whole body fatigability (Corwin *et al.*, 2002). The aetiology of this earlier onset of whole body fatigue in smokers is unknown and it is unclear as to whether smoking itself contributes to this phenomenon or other factors such as a decreased level of physical activity (Örlander *et al.*, 1979; Larsson & Örlander, 1984; Larsson *et al.*, 1988). In Chapter 3 we reported that in young male smokers, matched for physical activity with control subjects, smoking itself causes a significant decline in skeletal muscle fatigue resistance. The decline in fitness during prolonged smoking (Bernaards *et al.*, 2003) suggests, however, that long-term smoking may also cause a progressive deterioration of skeletal muscle fatigue resistance.

Here, we hypothesised that the reduction in skeletal muscle function and fatigue resistance would be related to the cumulative dose of smoking. Consequently we have examined the muscle fatigability of subjects with a range of exposures to smoking. To assess the effects of smoking duration on muscle function we have used electrically evoked contractions of the quadriceps muscle of otherwise healthy smokers. Electrical stimulation avoids possible motivational bias and also allows determination of contractile properties such as the force-frequency relationship and the rate of relaxation. The intermittent fatigue protocol used in this study mimics, to some extent, the rhythmic muscle contractions involved in activities of daily life such as cycling, walking and stair negotiation.

Methods and materials

Participants

Males and females were analysed separately, because of sex differences in fatigability (Chapter 2), smoking reportedly only affecting the experience of fatigue in men

(Corwin *et al.*, 2002) and differences between sexes in vulnerability to COPD (Gan *et al.*, 2006). Eighty-five people volunteered for the study after giving written informed consent. All procedures were approved by the local ethics committee and were in accordance with the Helsinki Declaration.

Table 4.1. Subjects characteristics. Values are displayed as mean ± SD (range)

	Male non-smokers	**Male Smokers**	**Female non-smokers**	**Female smokers**
n	22	19	23	21
Age (yr)	38.1 ± 17.8 (21 - 73)	37.0 ± 18.4 (18 - 72)	42.4 ± 20.4 (19 - 72)	37.5 ± 17.4 (18 - 66)
Body mass (kg)	77 ± 9 (63 – 95)	77 ± 12 (62 – 99)	66 ± 3 (53 - 83) **	74 ± 12 (56 – 96) **
BMI (kg m^{-1})	24 ± 2 (22-29)	24 ± 3 (18 – 30)	24 ± 3 (19 – 29)	27 ± 4 (22 - 34)
FVC (L)	5.4 ± 1.0 (3.6 – 7.3)	5.1 ± 1.3 (3.6 – 7.2)	3.6 ± 0.7 (2.5 – 4.8) **	3.7 ± 0.8 2.4 – 5.3) **
FEV_1 (L)	4.3 ± 1.0 (2.8 – 6.1)	4.0 ± 0.9 (2.6 – 5.6)	3.0 ± 0.6 (2.0 – 3.9) **	2.9 ± 0.8 (1.5 – 4.2) **
$\%FEV_{1pred}$	99.3 ± 13.2 (78.7 – 125.5)	91.0 ± 11.5 (81.0 – 111.8)	101.2 ± 14.7 (76.4 – 127.8)	92.0 ± 19.5 (47.6 – 119.9)
FEV_1/FVC (%)	78.7 ± 6.6 (60.2 – 91.6)	78.0 ± 9.4 (66.4 – 93.4)	81.7 ± 4.8 (71.9 – 88.6)	77.9 ± 10.5 (51.7 – 93.0)
Cigarette pack years	-	10.0 ± 12.8 (0.4 – 35.3)	-	9.8 ± 8.9 (0.4 – 28.0)
Physical activity score	8.2 ± 1.4 (6.0 – 10.8)	8.3 ± 1.4 (5.6 – 12.0)	8.1 ± 1.2 (5.8 – 9.5)	8.1 ± 1.4 (5.8 – 10.5)

No differences were observed between smokers and non-smokers, ** denotes P<0.005 from men. FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s in L or as a percentage of predicted

The smokers and non-smokers were matched for age and physical activity. Physical activity was assessed by questionnaire (Baecke *et al.*, 1982). Low values (< 6) represent a low physical activity level. Smoking history was assessed by questionnaire and smoking volume was determined by pack-years, which is defined as the number of cigarette packs smoked per day, multiplied by the number of years smoked. Exclusion

criteria were known respiratory, cardiovascular or neuromuscular diseases and leg injuries. Participants were asked to refrain from smoking and caffeine intake two hours prior to testing. The force expiratory volume in one second (FEV_1 (L)), predicted FEV_1 (%FEV_{1pred}), forced vital capacity (FVC) and FEV_1/FVC (%) were determined with standard spirometry (Vitalograph, Buckingham, England).

Measurement of anatomical cross sectional area (ACSA)

ACSA of the quadriceps muscle at 50 % of femur length was measured with magnetic resonance imaging (MRI) using a fixed 0.2 T MRI scanner (E-Scan, ESAOTE Biomedica, Genova, Italy). See the appendix for more details.

Measurement of maximal isometric voluntary knee extension strength

All knee extension torque measurements were performed on the right leg with a Cybex norm dynamometer (Ronkonkoma, New York, USA) with the hip joint at 90° flexion. For more details on the procedures, see the appendix and the *methods* section of chapter 2.

Voluntary activation

Beside muscle wasting, loss of strength can also be caused by a diminished ability to maximally activate the muscle. This ability was determined using the interpolated twitch technique (Shield & Zhou, 2004). Doublet pulses (pulse width 50 μs, 100 Hz; DSV Digitimer Stimulator, Digitimer Ltd., Herts, UK) were applied percutaneously using stimulation pads (Versastim, Conmed Corp., N.Y., USA) with the anode placed proximally and the cathode distally over the quadriceps muscle. The current required for supra-maximal stimulation was assessed by administering single pulses at rest. The ratio of electrically evoked force superimposed on an MVC to the force of a pre-contraction doublet provided an index of voluntary activation (VA). After the VA was determined the stimulation current was set at a level to produce approximately 30 % of MVC torque when stimulating at 100 Hz and this current was used throughout the rest of the experiment.

Torque-frequency relationship and contractile speed

The torque-frequency relationship and maximal rate of relaxation (MRR) both give an indication of the contractile speed and fibre type composition of the muscle (Scott *et al.*, 2006). The force-frequency relationship of the quadriceps muscle was assessed

by stimulating in random order at 1, 10, 15, 20, 30, 50 and 100 Hz for 1 s, each separated by 1 min. Differences in the torque–frequency curves were assessed using the 10/100 Hz ratio (FF_{10}/FF_{100}). The MRR was determined from the 50 Hz contraction (MRR_{50}) as peak dF/dt normalised to the maximal torque generated during that contraction.

Fatigue test

Five minutes after determining the torque-frequency relation, fatigue resistance was assessed by stimulating the quadriceps muscle with 30 Hz trains for 2 minutes (1 s on 1 s off). Torque and MRR for each contraction were expressed as a percentage of the first contraction. The percentage torque remaining after 2 minutes was defined as the fatigue index. The coefficient of variation between days was 2.3 and 5.3 % for the force-frequency relationship and fatigue test, respectively (n=3 non-smokers).

Statistical analysis

Differences between groups were tested using a 2 x 2 ANOVA with factors: smoke (non-smokers and smokers) and sex, with age as a covariant using SPSS (SPSS Inc., Chicago, IL, USA). The correlations between smoking habits and fatigability were performed using a partial correlation, correcting for age and gender. Differences were considered significant when $P<0.05$. Results are presented as means ± SEM unless otherwise stated.

Results

Subject characteristics

Table 4.1 shows the anthropometric characteristics of the participants. Age, physical activity levels, body mass, body mass index and lung function (FEV_1, FVC or FEV_1/FVC) were similar in smokers and non-smokers, although $\%FEV_{1pred}$ tended to be significantly lower in smokers (P=0.092). Although we did not check whether the airflow limitation in three smokers with an $FEV_1/FVC < 70$ % was reversible, this value suggests that they might have (undiagnosed) mild to moderate COPD (GOLD stage I or II). The inclusion of their data, however, did not alter the results of the present study.

Muscle strength, size and voluntary activation level

The VA was higher in the smokers (Table 4.2). Maximal torque capacity (MVC corrected for VA; MTC) was lower in women than men (Table 4.2) and decreased significantly in both men (R^2=0.43) and women (R^2=0.46) during ageing (P<0.001; Figure 4.1). If we consider smoking or non-smoking there was no difference in MTC and ACSA (Table 4.2). However, stepwise linear regression showed that height, age, gender, physical activity level and years smoked significantly predict the ACSA (R^2=0.793; P<0.001), while cigarette pack years and cigarettes smoked per day did not improve the model.

Table 4.2. Values for muscle strength, size and contractile properties.

	Male non-smokers	**Male smokers**	**Female non-smokers**	**Female smokers**
VA (%)	85.9 ± 3.0	92.0 ± 2.3 †	88.1 ± 2.2	90.0 ± 3.3 †
MVC (Nm)	286 ± 21	280 ± 18	190 ± 12 **	204 ± 15 **
MTC (Nm)	330 ± 18	300 ± 19	213 ± 12 **	219 ± 15 **
ACSA (cm^2)	72 ± 2	65 ± 3	53 ± 2	52 ± 2
FF_{10}/FF_{100}	31.3 ± 1.4	31.4 ± 3.0	33.3 ± 1.5 *	38.0 ± 2.0 *
MRR_{50} (s^{-1})	-15.1 ± 0.3	-16.0 ± 0.7	-12.7 ± 0.4 **	-12.9 ± 0.8 **

† P<0.05 from non-smokers; * P<0.05 from men; ** P<0.005 from men. VA: voluntary activation; MVC: maximal voluntary contraction; MTC: maximal torque capacity (maximal voluntary contraction corrected for VA); ACSA: anatomical cross-sectional area; FF_{10}/FF_{100}: force frequency relationship: torque produced at 10 Hz relative to 100 Hz; MRR_{50} : maximal rate of relaxation at 50 Hz.

Contractile properties

No differences were observed in the torque-frequency relationship or MRR_{50} between smokers and non-smokers (Table 4.2). Women however, had, on average, slower contractile properties than men as reflected by the less negative MRR_{50} and the higher FF_{10}/FF_{100} (Table 4.2).

Fatigue

Figure 4.2A and 4.2B show a typical example of the fatigue test (A), and an example of the reduction in torque and rate of relaxation from the first to the last contraction (B). In all groups there was a progressive decline in torque (Figure 4.3) and MRR,

the latter indicating a slowing of the muscle (Figure 4.4). Women were more fatigue resistant than men (P<0.001), both in terms of torque and MRR. In both men and women, the fatigue resistance was lower in smokers than non-smokers (P=0.014; Figure 4.3). The absence of a smoke x sex interaction indicates that the effect of smoking was similar in both sexes.

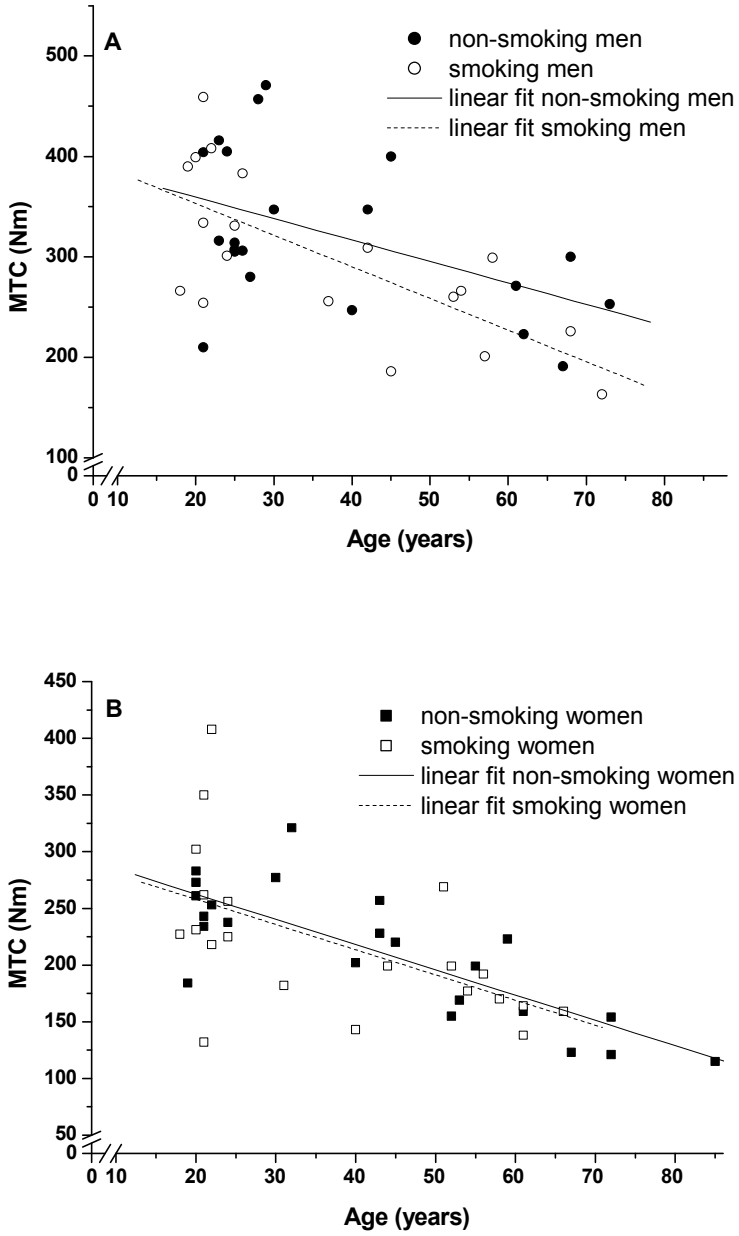

Figure 4.1. Maximal torque capacity (MTC), maximal voluntary torque corrected for voluntary activation of the quadriceps muscle expressed as a function of age in male (**A**) and female (**B**) smokers and non-smokers. MTC decreased significantly with age in men (R^2=0.43) and women (R^2=0.46; both P<0.001). The decrease in maximal strength over time (and smoke exposure) was similar between smokers and non-smokers (NS).

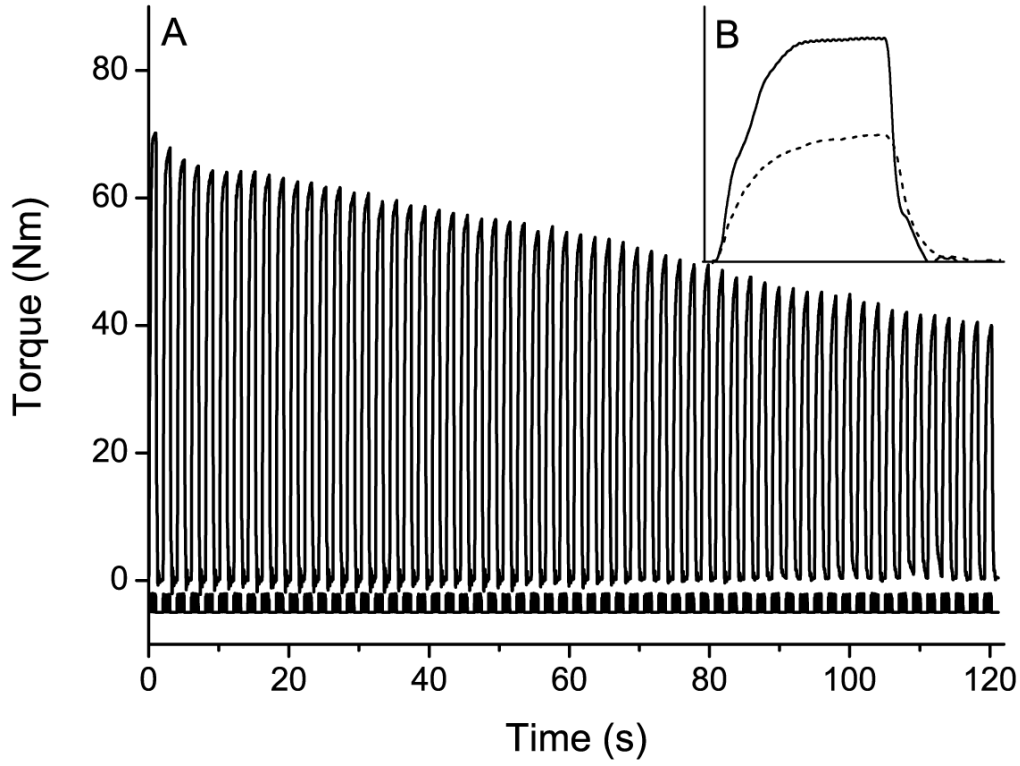

Figure 4.2. Representative example of a fatigue test (**A)**, with in **B** the first (continuous line) and last (dotted line) contraction enlarged.

The fatigue index in smokers was not related to cigarette pack years (Figure 4.5), packs smoked per day (r=-0.092, P=0.628), years smoked (r=0.242, P=0.321), physical activity level (r=-0.029, P=0.877), age (r=0.101, P=0.557) or lung function in terms of $\%FEV_{1pred}$ (r=-0.187, P=0.416) or FEV_1/FVC (r=0.03, P=0.824). Also, there was no relationship between lung function (FEV_{1pred}) and fatigue index (r=-0.027, P=0.921) when controlling for cigarette pack years, the number of smoking years and number of cigarettes smoked per day. Similar results were obtained for the decline in MRR. Stepwise linear regression revealed that gender (P=0.015) and being a smoker or non-smoker (P=0.024) explained 19.2 % of the variance in fatigue index between the participants.

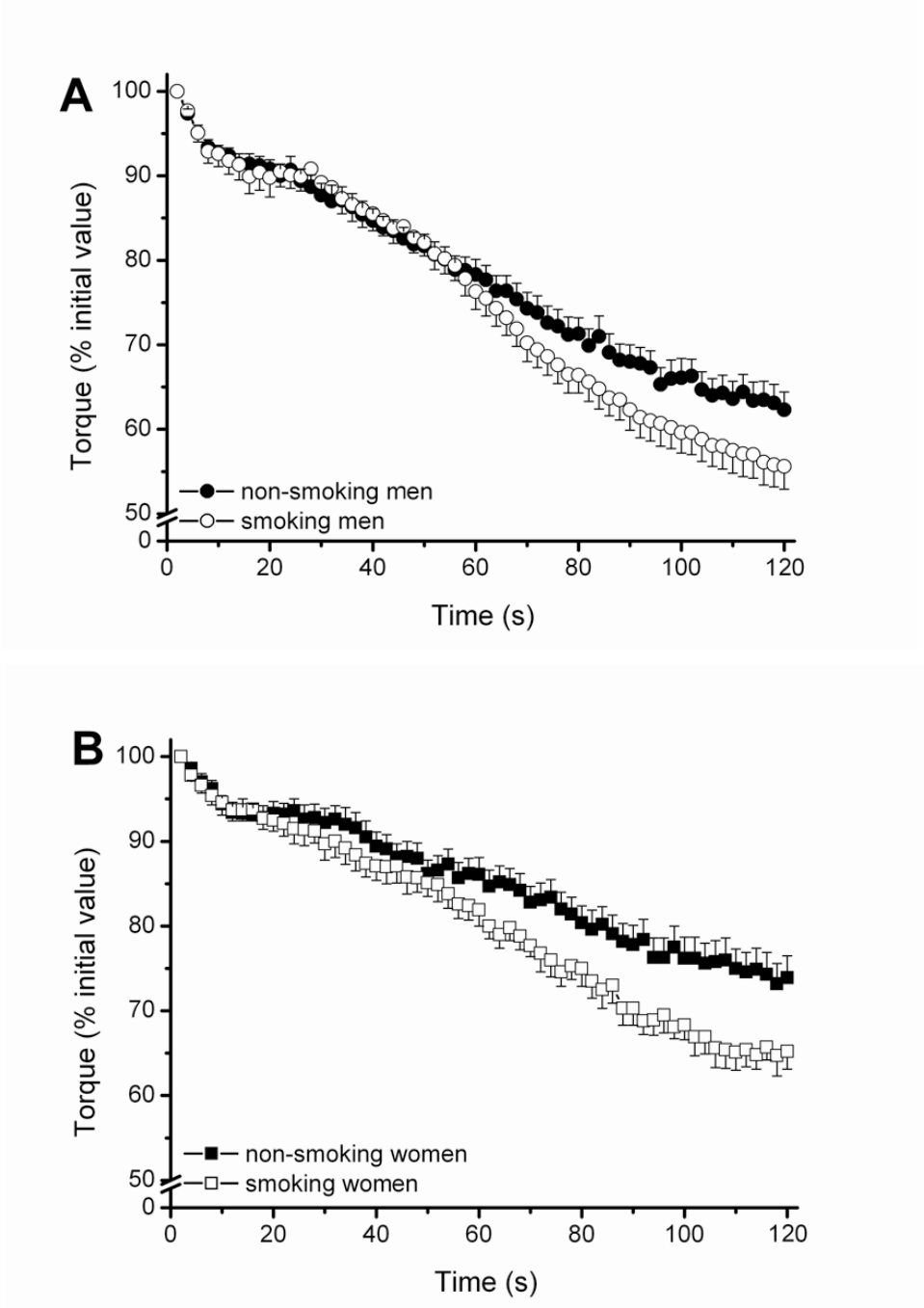

Figure 4.3. Torque, expressed as percentage of initial value, during the fatigue protocol is plotted every 2 s during the fatigue protocol for male (**A**) and female (**B**) smokers (open symbols) and non-smokers (closed symbols). Women had a significantly higher fatigue resistance ($P<0.001$). Both male and female smokers fatigued more than the non-smokers ($P=0.014$). Note that (**A**) is almost identical to Figure 3.2, but (**A**) includes more participants.

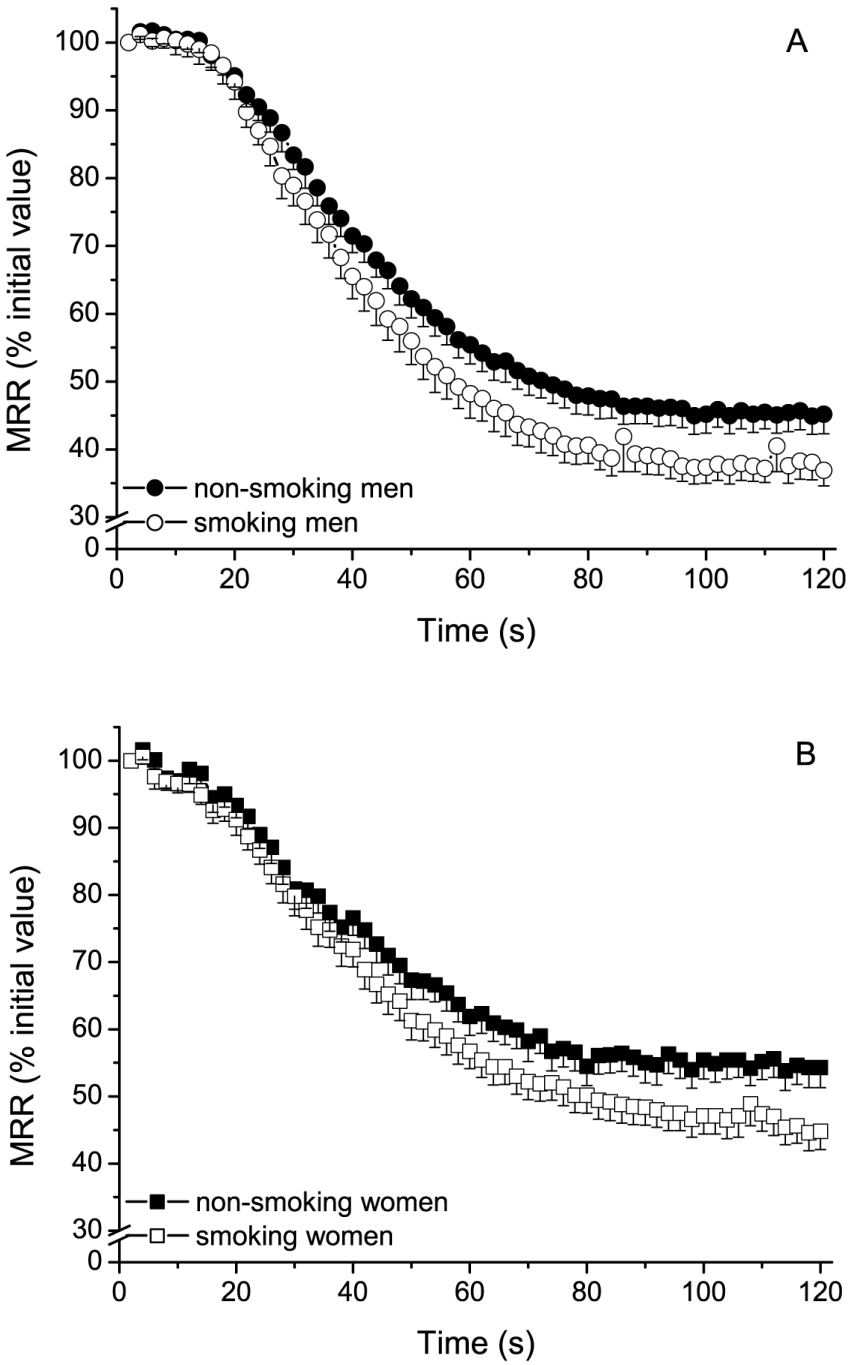

Figure 4.4. Maximal rates of relaxation (MRR), expressed as percentage of initial value, during the fatigue protocol are plotted every 2 s during the stimulation for male (**A**) and female (**B**) smokers (open symbols) and non-smokers (closed symbols). Significantly higher values were obtained in the females compared to the males ($P<0.001$). MRR decreased to a significantly lower level in the smokers compared to the non-smokers ($P=0.008$).

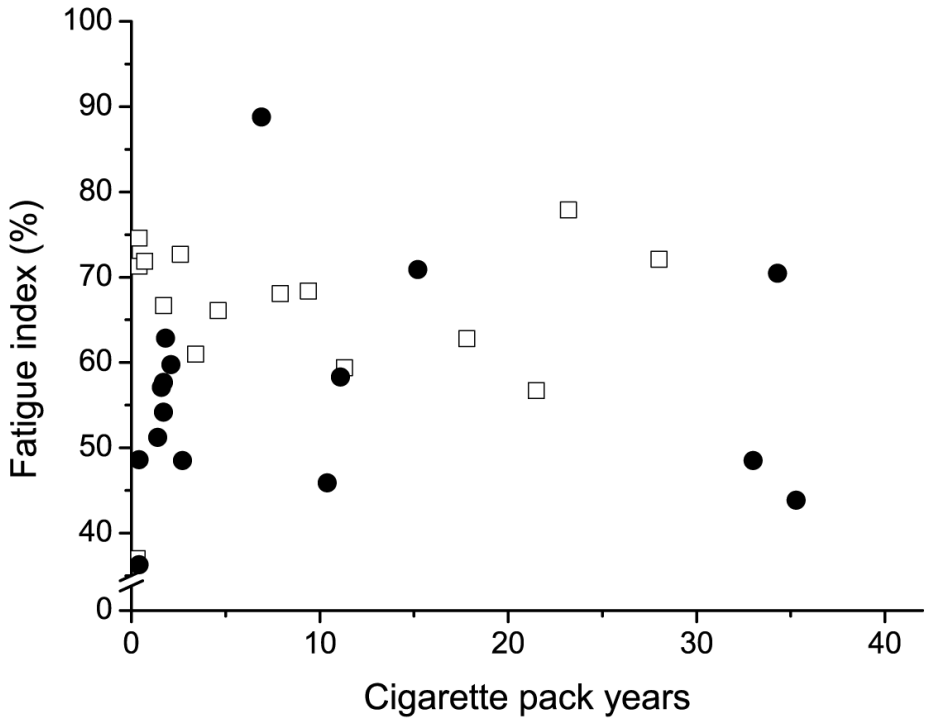

Figure 4.5. Relationship between cigarette pack years and fatigue resistance of the quadriceps muscle for male (●) and female (□) smokers of the fatigue test. Fatigue index was calculated as torque at the end of the test as a percentage of the initial value. The correlation coefficient was 0.095 (P=0.618) for fatigue index and similar results were obtained from other measures of smoking history (see text).

Discussion

We observed that smoking, in the absence of overt lung disease, is not accompanied by muscle weakness and wasting or changes in contractile properties of the quadriceps muscle after taking into account age, sex and physical activity. Despite an absence in changes in contractile properties and muscle weakness, the muscles of smokers were, however, more fatigable. This effect was similar in men and women. In chapter 3, we have shown that young smoking men show greater fatigability and here we hypothesised that the effect would increase with increasing smoking volume. The absence of correlations between muscle fatigability and any measure of smoking volume contradicts this hypothesis and suggests that the effect of smoking on skeletal muscle fatigability is of an acute nature and/or reaches a ceiling where an increased daily smoking volume does not aggravate the effect.

Maximal strength, size and voluntary activation

We did not find a lower maximal voluntary torque in smokers, despite a slightly higher voluntary activation level. The higher VA might be due to an increase in sympathetic nerve activity in smokers (Narkiewicz *et al.*, 1998), possibly due to a central stimulant action of nicotine (Mündel & Jones, 2006). Yet, this effect is insignificant as the maximal capacity of the muscle to generate torque (maximal torque corrected for VA) was also similar between smokers and non-smokers. Others (Örlander *et al.*, 1979; Al-Obaidi *et al.*, 2004), however, did report a lower maximal strength in smokers than non-smokers, but smokers and non-smokers were not matched for physical activity level, a factor very likely to affect maximal voluntary force. Other factors that may contribute to muscle weakness via muscle wasting are systemic inflammation and oxidative stress (Gan *et al.*, 2005; Wüst & Degens, 2007). The observation that the amount of years smoked predicted the ACSA of the quadriceps muscle suggests that long term smoking produces a small degree of muscle wasting. Surprisingly, we did not observe similar differences in the maximal strength of the quadriceps.

Contractile properties

The force-frequency relationship did not differ between smokers and non-smokers, suggesting that no significant, if any, shift in fibre type composition had occurred with smoking. Moreover, the MRR, a broad measure of muscle speed and fibre type composition (Hamada *et al.*, 2003), was not different between smokers and non-smokers. In line with our observation, it has been found that different doses of cigarette smoke did not lead to changes in fibre type distribution in the rat (Nakatani *et al.*, 2003). Örlander *et al.* (1979), however, found a lower percentage type I fibres in smokers, but this may be related to differences in physical activity level which was not controlled in their study.

Peripheral muscle fatigability

The main finding of the present study is a lower skeletal muscle fatigue resistance in smokers. Interestingly, the reduction in fatigue resistance was similar in men and women and, contrary to our hypothesis, was not related to several measures of smoking history. The increased fatigability could be caused by neuromuscular transmission failure, a shift to a more fatigable fibre type, reduction in oxygen supply

to the muscle and/or the oxidative capacity of the muscle (Degens & Veerkamp, 1994).

The likelihood of electrical failure either at the neuromuscular junction or T-tubules was minimised by keeping the stimulation frequency relatively low and allowing sufficient recovery intervals (Jones, 1996). Therefore, the site of fatigue is most likely distal to the neuromuscular junction (Bigland-Ritchie *et al.*, 1982).

Another factor possibly affecting muscle fatigue is the fibre type distribution of the muscle since type II fibres have a higher ATP turnover for the same isometric tension than type I fibres (Stienen *et al.*, 1996). Also, they generally have a lower oxidative capacity and fatigue more rapidly (Degens & Veerkamp, 1994). However, we did not observe any differences in maximal rate of relaxation or force-frequency relationship, both a qualitative *in vivo* suggestion of a different fibre type composition. It is thus unlikely that this explains the higher fatigability in smokers.

Another factor influencing muscle fatigue is the oxidative capacity of the muscle. Earlier studies have found that smokers have a lower activity of mitochondrial enzymes, such as cytochrome oxidase (Örlander *et al.*, 1979; Larsson & Örlander, 1984). The present study does not allow us to draw firm conclusions regarding oxidative capacity.

Impaired oxygen transport to skeletal muscle

The absence of a significant correlation between cigarette pack years and fatigability of the muscle and no indication for changes in fibre type composition that could explain the reduced fatigue resistance in smokers suggest that smoking acutely hampers the oxygen and/or energy delivery and/or utilization. An impaired oxygen delivery may occur if the blood flow is diminished or the oxygen content of the blood is lower than normal. Exercise-induced vasodilatation has been reported to be lower in smokers than physical activity matched non-smokers (Gaenzer *et al.*, 2001), which may be due to an impaired nitric oxide generation (Montes de Oca *et al.*, 2008) and/or an increased oxidative stress (Gaenzer *et al.*, 2001; Tsuchiya *et al.*, 2002).

A diminished oxygen delivery can also result from a reduced oxygen content of the blood. In smokers this could arise when the oxygen binding sites on haemoglobin (Hb) become occupied with carbon monoxide (CO), resulting in hypoxaemia. Indeed, carboxyhaemoglobin (COHb) may reach levels of up to 9 % in smokers

(Rietbrock *et al.*, 1992). Yet, we showed previously that subjects were no more fatigable in acute hypoxia when using the same protocol (Degens *et al.*, 2006). It therefore seems unlikely that hypoxaemia is the factor that distinguishes the smokers from the non-smokers. The effect of CO via its action on Hb can, however, not completely be excluded as, in contrast to hypoxaemia, the oxygen dissociation curve exhibits a shift to the left, inhibiting the release of oxygen from Hb (Rietbrock *et al.*, 1992). Indeed, inhalation of CO acutely impaired maximal oxygen consumption in both healthy participants (Aronow & Cassidy, 1975; Seppanen, 1977; Klausen *et al.*, 1983) and patients with COPD (Aronow *et al.*, 1977). Moreover, experimentally elevating blood COHb to 6 % in non-smoking men resulted in an increased fatigability in a test identical to our fatigue protocol (Morse *et al.*, 2008).

Besides the effect of CO on oxygen supply to the tissue, CO might also bind to Mb, limiting intra-cellular facilitated diffusion of oxygen. Also, CO blocks complex IV of the mitochondrial respiratory chain causing an overall decline in mitochondrial function (Alonso *et al.*, 2003). Cardellach *et al.* (2003) showed that mitochondrial respiratory chain function (complex III and IV [cytochrome oxidase]) in lymphocytes was decreased in smokers, but returned to normal values after smoking cessation.

Combined with the data in the present study this suggests that smoking may have an acute and reversible effect on skeletal muscle fatigability caused by CO (amongst other chemicals) in cigarette smoke. This then would result in a lower, but reversible, fatigue resistance in smokers. It should be noted, however, that not only CO, but also tar (Pryor *et al.*, 1992) and cyanide (Nelson, 2006) in the cigarette smoke could directly reduce mitochondrial respiratory chain function. This is further supported by the similar fatigue resistance (using the same fatigue test as in the present study) in non-smoking patients with COPD (Degens *et al.*, 2005).

In conclusion, skeletal muscle from smokers is more fatigable than that of age- and physical activity-matched non-smokers, while contractile properties of the skeletal muscle were not different. The decrease in fatigue resistance was similar in men and women and not related to smoking history. This suggests that smoking itself acutely affects skeletal muscle fatigue resistance. We speculate that this may occur via an acute and potentially reversible smoking-induced oxygen delivery to the working muscle and/or impairment of the function of haemoglobin and myoglobin due to

carbon monoxide. Moreover, CO and other factors in smoke inhibit enzymes in the respiratory chain (such as cytochrome oxidase). If smoking indeed has an acute effect on muscle fatigue resistance, smoking cessation may be helpful in the management of the exercise intolerance and sensations of fatigue in smokers, whether or not they suffer from clinical symptoms of chronic diseases such as COPD or heart failure. It may also help to break the vicious circle of disuse in order to prevent sensations of fatigue that in turn will cause a further decrease in physical fitness.

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CHAPTER 5

FIBRE CAPILLARY SUPPLY RELATED TO FIBRE SIZE AND OXIDATIVE CAPACITY IN HUMAN AND RAT SKELETAL MUSCLE

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Abstract

The capillary supply of a muscle fibre is thought to be determined by its type, oxidative capacity, size and metabolic surrounding. Size and oxidative capacity, however, differ between fibre types. To investigate which of these factors determines the capillary supply of a myofibre most we analysed in sections from human vastus lateralis (n=11) and rat plantaris muscle (n=8) the type, succinate dehydrogenase (SDH) activity, reflecting oxidative capacity, and capillary supply of individual fibres. Capillary fibre density differed between fibre types in rat ($P<0.03$) but not in human muscle. In human muscle only, the local capillary to fibre ratio (LCFR) correlated with the integrated SDH-activity (fibre cross-sectional area x SDH-activity) of a fibre ($r=0.62$; $P<0.001$). Backward multiple regression revealed, however, that the LCFR was primarily determined by fibre size, type ($r=0.71$, human) and surrounding of the fibre ($r=0.62$; rat plantaris muscle), i.e. whether it came from the deep or superficial region of the muscle (all $P<0.001$) and not SDH-activity. In conclusion, size, type and metabolic surrounding rather than mitochondrial activity determine the capillary supply to a muscle fibre.

Introduction

Since the pioneering work of Krogh (1919), many scientists have tried to understand the factors underlying the distribution of capillaries in skeletal muscle (Hoppeler *et al.*, 1981; Mathieu-Costello *et al.*, 1992; Degens *et al.*, 1994; Ahmed *et al.*, 1997). Oxidative muscles generally having a denser capillary network than glycolytic muscles (Saltin & Gollnick, 1983; Egginton, 1990; Degens *et al.*, 1992), but the capillary density of skeletal muscles varies widely between different species, muscles and even within a single muscle (Hoppeler *et al.*, 1981). Within the plantaris muscle for instance the deep, oxidative region has a denser capillary network than the superficial, glycolytic region (Degens *et al.*, 1992). The capillary supply to individual muscle fibres is thought to be determined by its oxidative capacity (Degens *et al.*, 1992; Mathieu-Costello & Hepple, 2002), size (Degens *et al.*, 1992; Ahmed *et al.*, 1997) and type (Saltin & Gollnick, 1983; Degens *et al.*, 1992). The main function of the capillaries is often thought to be oxygen supply, but removal of metabolites and heat and supply of substrates may be a more important function of the capillary bed. Thus, although all oxidative capacity, size and type of the fibre have been reported to be related to capillary supply, to what extent the capillary supply of an individual fibre is determined by each of these factors and how these relations may vary between human and rat is not systematically investigated.

Therefore, the primary aim of the present study is to quantify to what extent fibre type, oxidative capacity, fibre size and metabolic surrounding determine the capillary supply of an individual fibre, and whether or not these relations are qualitatively and quantitatively the same in rats and humans. As a measure for the oxidative capacity of a fibre we used sections stained for succinate dehydrogenase (SDH)-activity. It has been shown that using our methodology, the staining intensity of a muscle fibre for this enzyme from the citric acid cycle correlates very well with the maximum cellular oxygen uptake (Van der Laarse *et al.*, 1989), and hence the maximum demand for oxygen that has to be supplied by the surrounding capillaries.

Materials and methods

Rat biopsies

Eight male Wistar rats (11 weeks old) were killed by an intraperitoneal injection of an overdose of pentobarbital sodium and the plantaris muscles carefully excised, slightly stressed to minimise bias related to tortuousity of capillaries, directly frozen in liquid N_2 and stored at -80^{o}C until further analysis.

Human biopsies

Eleven healthy participants (5 men and 6 women, age: 42 ± 18 years) volunteered to participate in this study. Written informed consent was obtained from each participant prior to taking the percutaneous biopsy. All procedures were approved by the local ethics committee of the Manchester Metropolitan University (UK). After local anesthesia with 2 % lidocaine, a percutaneous biopsy of the vastus lateralis muscle (at ~50 % of the muscle length) was obtained using a conchotome. The biopsy was placed on cork, frozen in liquid N_2 and stored at -80^{o}C.

Histochemistry

Transverse 10 μm sections were cut in a cryostat at -20 ^{o}C. The sections were stored at -80 °C until further analysis, unless otherwise stated.

Fibres were classified as type I, IIa, or IIx (IIx/b in rats) based on the pH sensitivity of their myofibrillar ATPase, using the methods first described by Brooke & Kaiser (1970); type I myofibrillar ATPase is acid stable and alkaline labile, while type IIa myofibrillar ATPase is acid labile and alkaline stable with that of IIx/b in between. The fibres thus appear dark, white and grey after acid pre-incubation, respectively (Figure 5.1). Sections were pre-incubated for 10 minutes in a buffer containing 0.1 M NaAc and 0.1 M KCl at pH 4.55 set with acetic acid, quickly rinsed in 20 mM glycin and 20 mM $CaCl_2$ at pH 9.4 (set with NaOH) and subsequently incubated for 25-30 minutes at 37°C in 40 mM glycin, 20 mM $CaCl_2$ and 2.5 mM ATP at pH 9.4 (set with NaOH). Sections were quickly rinsed with 1 % $CaCl_2$, left in 2 % $CoCl_2$ for 3 minutes, washed with distilled water and coloured in 2 % $(NH_4)_2S$ and washed in water.

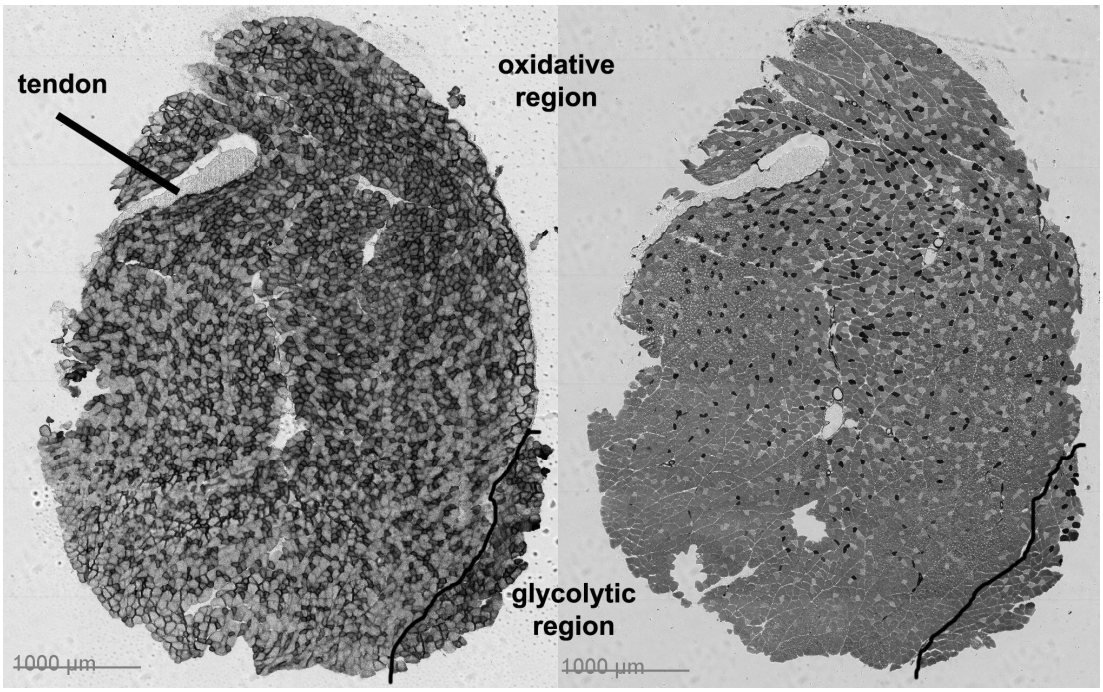

Figure 5.1. Example of a plantaris muscle of the Wistar rat. On the left, the section is stained for SDH-activity (see text), while the right serial section was stained for acid myofibrillar ATPase (pH=4.55). The dark fibres on the right are classified as type I, while the white fibres are classified as type IIa and the grey fibres as type IIx/b. One can observe that the type I and IIa fibres have, as expected, a higher SDH-activity. Note that on the right hand corner of the muscle, a small part of the gastrocnemius muscle is still visible.

A serial section was stained for SDH-activity (Van der Laarse et al., 1989; Figure 5.1). Succinate dehydrogenase (SDH) is an enzyme in the citric acid cycle and part of the respiratory chain. It has been shown by our lab that the calibrated histochemical method described below is linearly related to the maximum rate of oxygen uptake by the muscle fibre (Van der Laarse *et al.*, 1989). Immediately after cutting, sections were dried for 15 min and stained for SDH-activity (Van der Laarse *et al.,* 1989; Pool *et al.*, 1979). Thereto, the sections were incubated at 37°C in the dark for 20 min in a medium consisting of 37 mM sodium phosphate buffer (pH 7.6), 74 mM sodium succinate and 0.4 mM tetranitroblue tetrazolium (TNBT). The reaction was stopped with a 30 s incubation in 0.01 M HCl and fixed in 10 % formalin for 20 min. The staining intensity was determined as the optical density of the final reaction product using an interference filter of 660 nm at a magnification of x 20 (ImageJ, National Institute of Health, Bethesda, USA) and was calculated as the absorbance at 660 nm per µm section thickness per second of staining time (ΔA_{660} μm^{-1} s^{-1}).

Another serial section were stained with alkaline phosphatase (rat; Degens et al., 1992) or lectin (human; Ahmed et al., 1997) to depict capillaries. In short, rat sections were fixed in chloroform-aceton (1:1; 4°C), quickly rinsed in distilled water and subsequently stained for 1 h in 0.01 % nitro blue tetrazolium (NBT) and 0.002 % 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt in 6.9 mmol $MgSO_4.7H_2O$ and 27.5 mmol $Na_2B_4O_7.10H_2O$ buffer adjusted to pH 9.2 with boric acid. After quickly rinsing the sections in distilled water and a postfixation in 10% formalin (4 % formaldehyde), slides were mounted in gelatin-glycerol.

Human sections were fixed in acetone (4°C), kept in HEPES for 15 minutes, incubated in PBS containing 0.9 % 30 % H_2O_2 for 30 minutes to reduce background staining and subsequently incubated with lectin (*Ulex Europeus*) in HEPES with 1 % BSA (2000 µg/ml). The capillaries were visualised using a Vectastain ABC and DAB substrate kit (Vector Laboratories, Peterborough, UK) before mounting in gelatin-glycerol.

Determination of capillarisation

The capillarisation was analyzed with the method of capillary domains (Hoofd et al., 1985; Degens et al., 1992). In short, domains were constructed around capillaries, defined as the area surrounded by a capillary delineated by equidistant boundaries from adjacent capillaries. The fibre cross-sectional area (FCSA) was determined by tracing the outlines of the fibres on the digitizing tablet. The capillary supply to a fibre was given as the local capillary to fibre ratio (LCFR), calculated as the sum of the fractions of the capillary domains overlapping a given fibre.

Statistics

A 3x3 ANOVA was performed with as factors fibre type and muscle origin using SPSS (SPSS Inc., Chicago, IL, USA). If interactions were found, a repeated measures ANOVA (with Bonferroni post-hoc tests) was done to detect differences between fibre types within a muscle or muscle region, and an ANOVA (with Bonferroni post-hoc tests) for muscle origin was done to detect whether a certain fibre type differed between human and rat deep or superficial region. To test which factor predicted the capillary supply of the muscle most, backwards multiple regression analysis was performed. Correlations and differences were considered significant at $P \leq 0.05$. Data are presented as mean ± SD.

Results

Per animal/human, 45 ± 7 fibres were analysed for the deep plantaris region, 70 ± 18 fibres for the superficial plantaris and 40 ± 10 fibres for the human quadriceps. The results from fibre size, capillary supply and oxidative capacity are given in Table 5.1. The overall capillary to fibre ratio (C/F) and capillary density (CD) are shown in Table 5.1 under '*Total*'. In line with previous observations (Bekedam *et al.*, 2003) we found a significant correlation between the LCFR and SDH x FCSA in human ($r=0.62$; $P<0.001$), but not in rat muscle. However, backward multiple regression revealed that the local capillary to fibre ratio was not determined by SDH-activity, but determined by fibre size and type in the human vastus lateralis muscle ($r=0.71$) and also surrounding of the fibre ($r=0.62$; rat plantaris muscle); i.e. whether it came from the deep or superficial region of the muscle (all $P<0.001$). Figure 5.2A shows the positive correlation between LCFR and FCSA for each muscle.

There was even a negative relation within fibre types between LCFR and capillary fibre density (CFD=LCFR/FCSA) with SDH-activity of a fibre, as shown in Figure 5.2B and C for the deep region of the rat plantaris muscle. However, if we controlled for FCSA and fibre type, the correlations between SDH-activity and capillarisation disappeared ($r<0.1$, NS). As mitochondria are not homogenously distributed within a muscle cell we plotted the mitochondrial distribution against the fraction of the intercapillary distance, where capillaries were located at opposite sites of the muscle cell. The mitochondrial density was highest in close proximity of the capillaries (subsarcolemmal mitochondria) and exhibited a steep decline to the centre of the myofibre (interfibrillar mitochondria) in the oxidative type I and IIa fibres. Furthermore, the profile of the mitochondrial density was flatter in glycolytic type IIx/b than in the more oxidative type I and IIa fibres (Figure 5.2 D-F). Where in human muscle type IIx fibres have a higher LCFR than type I and IIa fibres, the opposite was found in rat muscle. This apparent discrepancy is largely related to fibre size (Table 5.1). To take this into account we also

Table 5.1. Indices of fibre size and capillary supply in the human vastus lateralis (VL) and the deep and superficial region of the plantaris muscle of male Wistar rats.

	Area %	FCSA (μm^2)	LCFR	CFD (mm^{-2})	SDH-activity ($\Delta A_{660}\ \mu m^{-1}\ s^{-1}$)
Human VL					
I	$52.2 \pm 5.4^{\#+\dagger}$	$4068 \pm 1300^{\#+}$	$1.51 \pm 0.68^{+\dagger}$	$362 \pm 145^{\#}$	$1.64 \pm 0.12 \times 10^{-5\,+\dagger}$
IIa	$45.3 \pm 5.3^{\#+\dagger}$	$3754 \pm 1555^{\#+}$	$1.33 \pm 0.70^{+}$	$325 \pm 138^{\#}$	$1.34 \pm 0.22 \times 10^{-5\,+\dagger}$
IIx	$2.5 \pm 1.9^{\#+}$	3669 ± 1382	$0.90 \pm 0.69^{\#}$	$254 \pm 105^{\#}$	$1.28 \pm 0.12 \times 10^{-5\,+}$
Total (n=426)		$3897 \pm 1387^{\#}$	1.33 ± 0.67	$349 \pm 140^{\#}$	$1.42 \pm 0.10 \times 10^{-5\,\#}$
Rat plantaris, deep region					
I	$8.8 \pm 4.7^{+\Delta\dagger}$	$1694 \pm 218^{\dagger}$	$1.28 \pm 0.35^{+\dagger}$	$765 \pm 245^{+\Delta\dagger}$	$1.26 \pm 0.55 \times 10^{-5\,+\dagger}$
IIa	$24.3 \pm 5.3^{\dagger}$	$1998 \pm 452^{\dagger}$	$1.24 \pm 0.25^{\dagger}$	$640 \pm 148^{+\dagger}$	$1.57 \pm 0.69 \times 10^{-5\,+\dagger}$
IIx/b	66.8 ± 8.3	3116 ± 641	1.81 ± 0.37	$613 \pm 174^{+}$	$0.87 \pm 0.49 \times 10^{-5\,+}$
Total (n=571)		$2581 \pm 523^{+}$	$1.55 \pm 0.31^{+}$	$638 \pm 170^{+}$	$1.14 \pm 0.49 \times 10^{-5\,+}$
Rat plantaris, superficial region					
I (n=5)	$0.8 \pm 1.0^{\Delta\dagger}$	$1271 \pm 266^{\dagger}$	$0.56 \pm 0.26^{+\dagger}$	$494 \pm 268^{\dagger}$	$1.04 \pm 0.49 \times 10^{-5\,\dagger}$
IIa	$14.4 \pm 20.0^{\dagger}$	$2174 \pm 1103^{\dagger}$	$0.76 \pm 0.33^{\dagger}$	395 ± 119	$0.88 \pm 0.48 \times 10^{-5\,\dagger}$
IIx/b	83.8 ± 20.2	3605 ± 739	1.09 ± 0.23	329 ± 58	$0.50 \pm 0.38 \times 10^{-5}$
Total (n=347)		3453 ± 449	1.07 ± 0.16	334 ± 55	$0.50 \pm 0.23 \times 10^{-5}$

VL: vastus lateralis; FCSA: fibre cross-sectional area; LCFR: local capillary to fibre ratio; CFD: capillary fibre density. $^{\#}$: significantly different from rat plantaris deep region at $P<0.02$; $^{+}$: significantly different from rat plantaris superficial region at $P<0.02$; $^{\Delta}$: different from type IIa of same origin at $P<0.05$; †: different from type IIx (or IIx/b) of same origin at $P<0.05$.

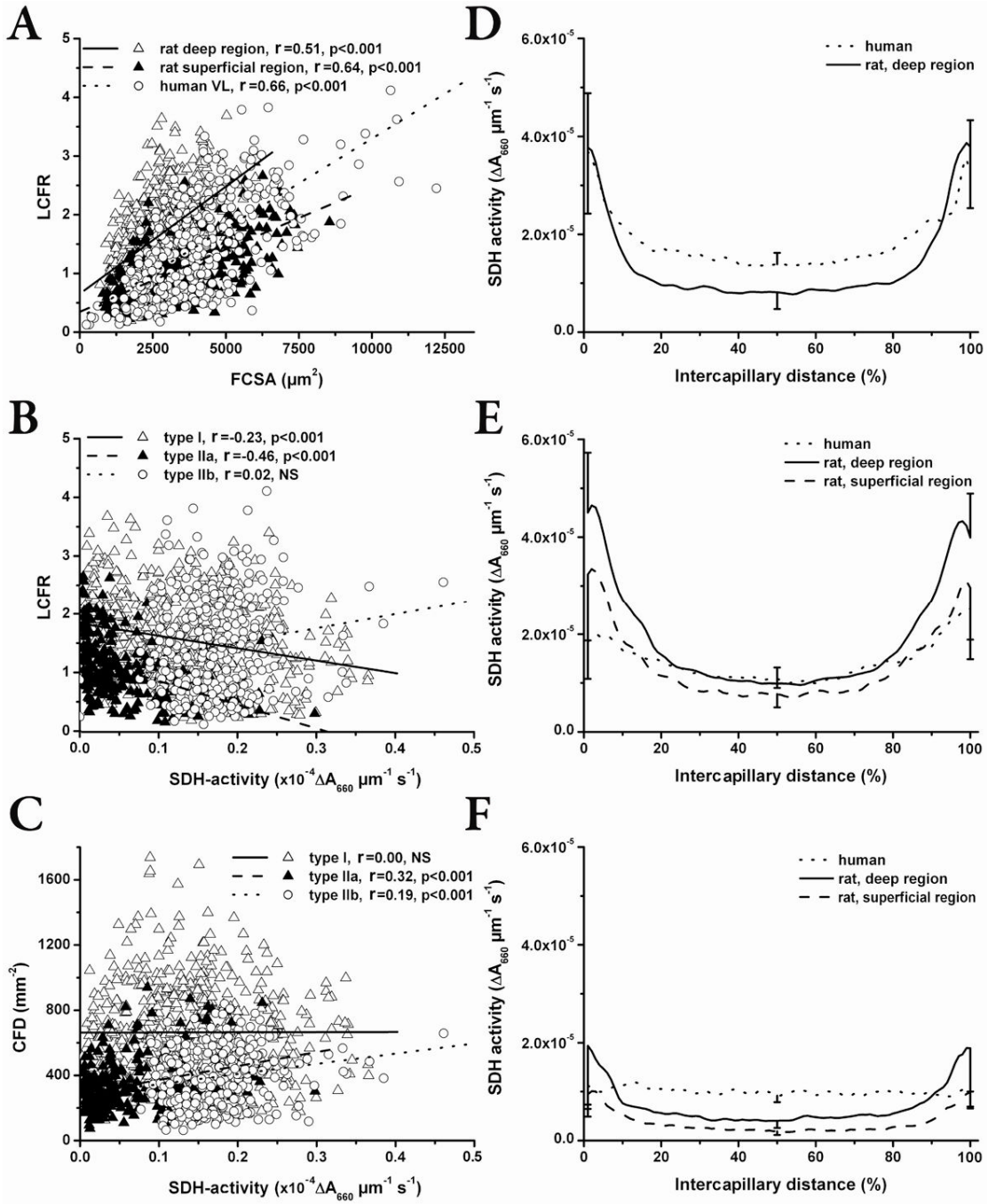

Figure 5.2. Relationships between (**A**) FCSA and LCFR for rat deep region, rat superficial region and human vastus lateralis, (**B**) SDH-activity and LCFR and (**C**) SDH-activity and CFD for the deep region only. SDH-activity between two capillaries are shown in **D-F** (**D**: type I, **E**: type IIa, **F**: type IIx (IIx/b)).

determined the CFD for each fibre type and it appeared that type I fibres had a higher CFD than type IIx/b fibres in both the deep and superficial region of the rat plantaris muscle (Table 5.1), but not in human muscle.

Table 5.1 and Figure 5.1A show that the relation between LCFR and FCSA is not fixed, but differs between human and rat muscle and also between the deep and

superficial region of the rat plantaris muscle. In terms of CFD, the capillary supply to a fibre is highest in the deep region of the plantaris muscle and no significant difference was observed between the CFD of fibres from the human vastus lateralis muscle and the superficial region of the rat muscle, even though the SDH-activity of the human fibres was higher.

Discussion

The main observation of the present study is that in both human and rat muscle the capillary supply to a fibre is primarily determined by its type and size, while the oxidative capacity plays no significant role.

Many studies have shown a significant relation between capillarisation and oxidative capacity in muscle tissue (Saltin & Gollnick, 1983). Also the capillary supply to individual fibres has been reported to be related to mitochondrial volume or integrated SDH-activity, in hummingbirds (Mathieu-Costello *et al.*, 1992) and humans (Bekedam *et al.*, 2003). As there is a hyperbolic relationship between fibre size and SDH-activity (Van der Laarse *et al.*, 1998), correlations between capillary supply and oxidative capacity may be confounded by fibre size. Indeed, it has been observed in both rat (Degens *et al.*, 1994) and human muscle (Ahmed *et al.*, 1997) that size is an important determinant of capillary supply to a fibre. Here we took into account both fibre size and SDH-activity and found that not SDH-activity, but fibre size was an important determinant of capillary supply to a fibre in both rat and human muscle (Figure 5.1A). Moreover, in rat muscle (integrated) SDH-activity did not correlate significantly with capillary supply to a fibre. Thus, taking into account both fibre size and oxidative capacity it appeared that size, rather than oxidative capacity is a significant predictor of the local capillary to fibre ratio.

Although Ahmed *et al.* (Ahmed *et al.*, 1997) suggested that there was no effect of fibre type we did observe with backward regression that also the type of the fibre had an impact on the capillary supply of a fibre. Nevertheless, in line with what we found they observed that the average LCFR was type I > IIa > IIx/b. The effect of type is, however, rather small, as both Ahmed *et al.* (1997) and we observed that the CFD, which takes into account the size of the fibre, was similar in each fibre type. In the rat plantaris muscle, on the other hand, the CFD of type IIx/b fibres was lower than that of type I and IIa fibres, but this difference was not explicable by differences in

SDH-activity between fibre types as revealed with backward regression for type and SDH-activity. Even the spatially integrated SDH-activity, as a measure for the total demand for O_2 of the myofibre (Bekedam *et al.*, 2003), did not significantly correlate with the capillary supply to a fibre in the rat plantaris muscle.

As the mitochondria ultimately consume O_2 one would have expected that the oxidative capacity is the main determinant of the capillary supply to a fibre. It should be noted, however, that mitochondria are not homogenously distributed within the fibre, where the density is highest at the sarcolemma, decreasing steeply to the interior of the cell (Hoppeler *et al.*, 1981). Here, we confirm that observation and show that the density distribution shows a flatter profile in human than rat fibres and in type IIx/b than type I and IIa fibres. Such a profile would considerably shorten the diffusion distance to the majority of the mitochondria, and hence result in an adequate O_2 supply to the mitochondria. This would, at least partly, explain why there is no significant relation between the oxidative capacity of a fibre and its capillary supply.

Finally, in line with previous observations (Degens *et al.*, 1992) we observed that the capillary supply to a fibre is modulated by the metabolic surrounding of the fibre. This is reflected by the different slope of the FCSA-LCFR relation and the lower CFD for each fibre type in the superficial than the deep region of the muscle.

In conclusion, in both rat and human muscle size and type rather than mitochondrial activity is the primary determinant of capillary supply to a muscle fibre.

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CHAPTER 6

SKELETAL MUSCLE CAPILLARISATION AND OXIDATIVE METABOLISM IN HEALTHY SMOKERS

BRIEF COMMUNICATION

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Abstract

We investigated whether the lower fatigue resistance in smokers than non-smokers is caused by a compromised muscle oxidative metabolism. Using calibrated histochemistry, we found no differences in succinate dehydrogenase (SDH)-activity, myoglobin concentration and capillarisation in sections of the vastus lateralis muscle of five smokers and five non-smokers. The relationship between fatigue resistance and SDH-activity in non-smokers ($r=0.93$, $P=0.02$) is absent in smokers. This indicates that the lower muscle fatigue resistance of smokers is likely due to other causes than differences in oxidative metabolism and local capillarisation.

Introduction

Chronic obstructive pulmonary disease (COPD) is associated with a reduced exercise tolerance and, in general, reduced muscle fatigue resistance (Gosselink & Decramer, 1998; Chapter 1). The main determinants of skeletal muscle fatigue resistance are: oxygen delivery to the muscle, oxygen transport from interstitial space to the core of the muscle fibres, fibre type composition and the oxidative capacity (Degens & Veerkamp, 1994), which is proportional to succinate dehydrogenase (SDH) activity (Van der Laarse *et al.*, 1989). The oxygenation of muscle tissue is largely dependent on capillary density (Hoofd *et al.*, 1985), myoglobin concentration (Richardson *et al.*, 2001) and fibre cross-sectional area (Hill, 1965). In patients with COPD, muscle fibre atrophy and a reduction in capillarisation and oxidative capacity all negatively affect muscle fatigue resistance (Maltais *et al.*, 1996; Jobin *et al.*, 1998; Green *et al.*, 2008; Chapter 1). As cigarette smoking is associated with over 80 % of the COPD cases (Yawn & Kaplan, 2008), smoking may be an important factor in the muscle adaptations that compromise the strength and fatigue resistance of COPD patients; skeletal muscle fatigue resistance is already lower in young, healthy smokers than age- and physical activity-matched non-smokers (Chapter 3). Although inhaled carbon monoxide (CO), a constituent of cigarette smoke, that reached 6 % carboxyhemoglobin (COHb) resulted in a 8 % reduction in the fatigue resistance of non-smoking subjects (Morse *et al.*, 2008), it is not clear whether this is the sole explanation. Given the determinants of skeletal muscle fatigue resistance described above, we hypothesised that in skeletal muscle mitochondrial volume density, capillary density and/or myoglobin concentration would be lower in smokers, and/or that the proportion of fast muscle fibres would be higher in the skeletal muscle of smokers than of non-smokers.

Methods

Participants

Five non-smokers (2 men and 3 women) and 5 smokers (2 men and 3 women) participated in this study. Exclusion criteria were known cardiovascular, neuromuscular or respiratory diseases. Written informed consent was obtained from

each participant prior to testing. All procedures were approved by the local ethics committee of the Manchester Metropolitan University (UK). The age of the non-smokers (range: 23-72 years, median: 45 years) and smokers (range: 25-72 years, median: 40 years) was similar. The smokers smoked on average 15 ± 9 cigarettes d^{-1}, while the non-smokers never smoked. Mean cigarette pack years (packs smoked d^{-1} x the number of years of smoking) was 12.9 (range: 2.6-35.0, median: 9.9) pack years. The physical activity score of the subjects assessed by questionnaire (Baecke *et al.*, 1982), was 8.1 ± 1.3 and 7.1 ± 1.2 for the non-smokers and smokers, respectively (P=0.20). A physical activity score of < 6 indicates a sedentary lifestyle, whereas scores > 9 represent a high level of activity.

Maximal strength, fatigue resistance and anatomical cross-sectional area

Maximal strength of the quadriceps muscle was determined as the highest value during a maximal voluntary contraction at optimal knee angle (for more details, see Chapter 2, 3 and 4). Fatigue resistance of the quadriceps muscle was assessed using electrical stimulation (1 s on 1 s off, 30 Hz, 2 min; constant stimulus intensity corresponding to 22 ± 4 % of maximal strength in the non fatigue state). Anatomical cross sectional area (ACSA) was measured at 50 % of femur length with magnetic resonance imaging (MRI). The procedures are detailed in chapter 2, 3 and 4 and in the appendix.

Skeletal muscle biopsy

A percutaneous biopsy of the vastus lateralis muscle was obtained from each participant using a conchotome. The site of the biopsy was anesthetised with 2 % lidocaine and a 1-cm incision was made at ~20 cm above the patella. The biopsy was frozen in liquid nitrogen and stored at -80 ^{o}C.

Enzyme histochemistry

Transverse 10 µm sections were cut in a cryostat at -20 ^{o}C. Sections were stained for myofibrillar adenosine triphosphatase (mATPase), after pre-incubation at pH 4.7, to classify fibres as type I or type II (Brooke & Kaiser, 1970, Chapter 5). Serial sections were incubated for myoglobin concentration (Van Beek-Harmsen *et al.*, 2004) and SDH-activity (Bekedam *et al.*, 2003, Chapter 5), which is proportional to the maximum rate of oxygen consumption when oxygen is not rate-limiting (Van der

Laarse *et al.*, 1989). SDH-activity is given as the absorbance increase at 660 nm per micrometer section thickness and per second of incubation time (ΔA_{660} μm^{-1} s^{-1}).

The myoglobin concentration in individual muscle fibres was determined as described previously (Van Beek-Harmsen *et al.*, 2004). Frozen sections were defrosted in vacuum to avoid condensation and consequent redistribution of myoglobin. Sections were then vapour-fixed for 1 h in paraformaldehyde at 70°C. Subsequently, the sections were fixed for 10 min at room temperature in 2.5 % glutaraldehyde in 0.07 M sodium phosphate buffer (pH=7.4) and quickly rinsed with distilled water. To detect myoglobin sections were incubated for 1 h at room temperature in a medium containing 59 ml 50 mM TRIS/80 mM KCl buffer (pH=8.0), 25 mg ortho-toluidine dissolved in 2 ml 96 % ethanol (at ~50°C) and 1.43 ml 70 % tertiary-butyl-hydroperoxide. Sections were then washed with distilled water, mounted and scanned on a calibrated DMRB microscope (Leica, Wetzlar, Germany) with an interference filter of 436 nm. A 3rd order calibration line was obtained before scanning each section using grey filters with a known absorbance.

The red blood cells in the capillaries contain haemoglobin which was visible as dark spots at the edges of the muscle fibres. The myoglobin concentration was, however, homogeneously distributed within the cell and therefore the mean optical density was obtained from the centre of each fibre. After subtracting the optical density of the background (a part of the section that did not contain myoglobin), optical density values were converted to absorbance values. Using a calibration line, obtained from absorbance values from 10 µm sections cut from a gelatin block containing 0.1, 0.2, 0.3 and 0.4 mM myoglobin, absorbance values were converted into concentrations (Van Beek-Harmsen *et al.*, 2004).

Capillarisation

Another serial section was stained with lectin to depict capillaries (Ahmed *et al.*, 1997, Chapter 5). Capillarisation was analysed with the method of capillary domains described by Degens *et al.* (1992). This method gives not only the overall indices of capillary density and capillary to fibre (C/F) ratio, it also takes into account the presence of different fibre types and the heterogeneity in capillary spacing (standard deviation of the log-transformed radius of the capillary domain ($Log_R SD$)). Domains were defined as the area surrounded by a capillary delineated by equidistant boundaries from adjacent capillaries. The fibre cross-sectional area (FCSA) was

determined by tracing the outlines of the fibres on a digitizing tablet (Model MMII 1201, Summagraphics Digitizers, Austin, Tex, USA). The capillary supply to a fibre was given as the local fibre to capillary ratio (LFCR), calculated as the sum of the fractions of the capillary domains overlapping a given fibre. The capillary fibre density (CFD in mm^{-2}) was calculated as the LCFR/FCSA.

Statistical analysis

We used multilevel analyses to evaluate differences between smokers and non-smokers (MLwiN version 2.0, Bristol, UK). This method incorporates nominal variables (Twisk, 2006), as well as variance at the level of the cell and the individual. Slow and fast fibres were analysed separately and gender, age and physical activity were included as covariates. Significance level was set at $P<0.05$. Unless otherwise stated, values are given as means ± standard error of the mean (SEM).

Results

The correlation coefficient between the physical activity score and the fibre-type weighted mean SDH-activity was 0.87 for non-smokers (P=0.06) and 0.88 for smokers (P=0.01). All participants had a normal respiratory function (data not shown).

Maximal strength and fatigue resistance

Maximal torque and ACSA were not different in non-smokers and smokers, with 201 ± 24 *vs.* 203 ± 35 Nm (P=0.96) and 61 ± 4 *vs.* 54 ± 6 cm^2 (P=0.32) for torque and ACSA respectively. Fatigue resistance, however, was significantly reduced by smoking: torque dropped to 77.4 ± 4.5 *vs.* 61.4 ± 4.5 % of initial value in non-smokers and smokers, respectively ($P<0.05$). The correlation coefficient between physical activity levels and fatigue index was 0.83 (P=0.08) and -0.88 (P=0.05) for the non-smokers and smokers, respectively. No significant relationship was observed between cigarette smoking history and the fatigue index: correlation coefficients were 0.73, -0.32 and 0.45 for cigarettes d^{-1}, years of smoking and cigarette pack years, respectively.

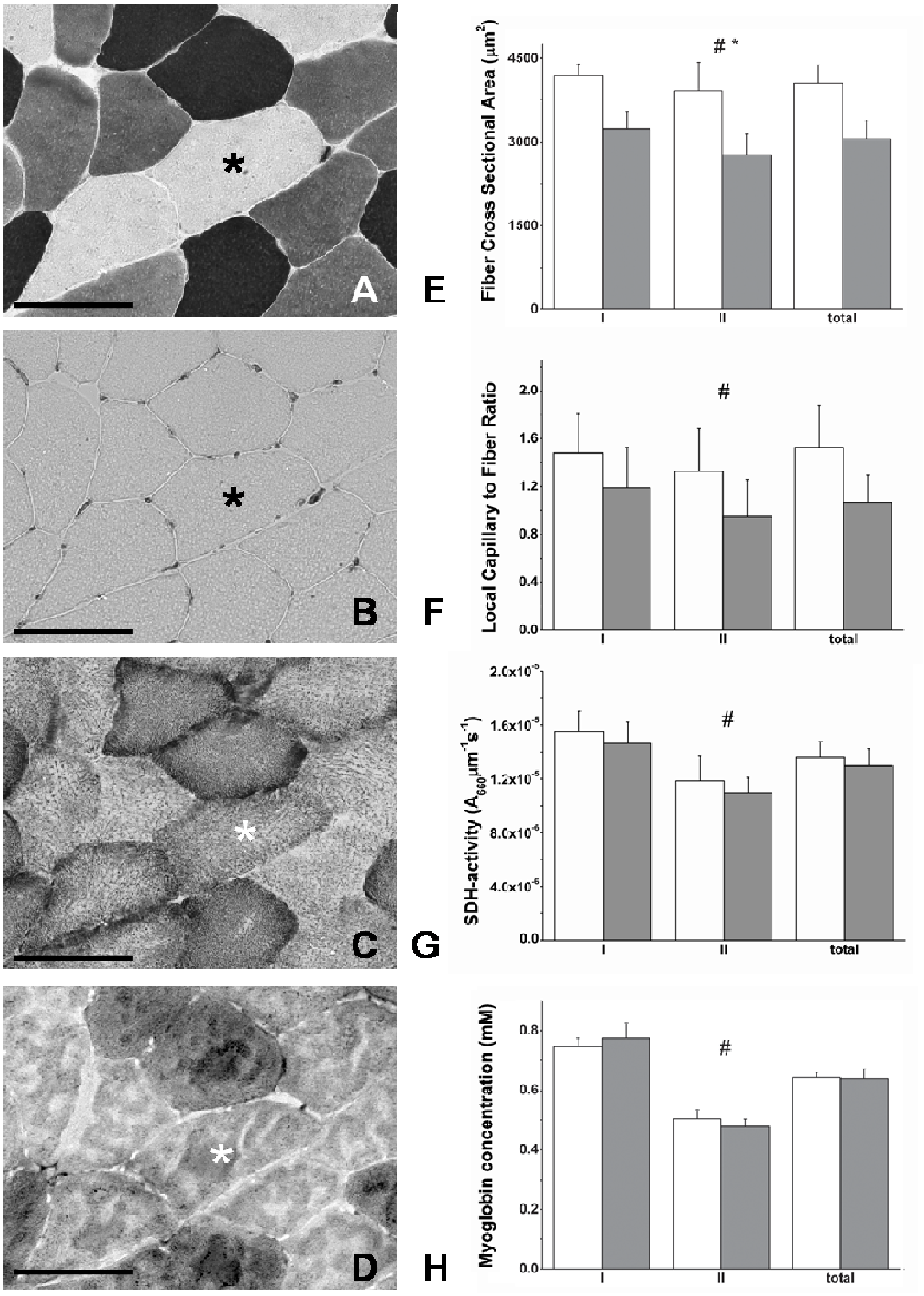

Figure 6.1. Serial cross-sections of a representative sample and results of fibre types and fibre cross-sectional area (**A** + **E**), capillary staining and local capillary to fibre ratio or capillary to fibre ratio (for 'total'; **B** + **F**), calibrated succinate dehydrogenase (SDH)-activity (**C** + **G**) and myoglobin concentration (**D** + **H**). The white bars represent the non-smokers and the grey bars represent the smokers. Total is the weighted average of the type I and II cells. Bars in photomicrographs indicate 100µm and * indicate the same fibre. In **A,** black is type I, white is type IIa and grey is type IIx. Values are mean (SEM). In **E-H**, # and * denote statistical differences between type I and II and between smokers and non-smokers respectively.

Muscle biopsies

On average 160 (SD: 45) fibres were analysed for each subject. Figure 1 shows examples of mATPase staining (Figure 6.1A), capillaries (Figure 6.1B), SDH-activity (Figure 6.1C) and myoglobin concentration (Figure 6.1D). The fibre type composition was similar in smokers and non-smokers, with 55 ± 4 *vs.* 59 ± 11 % and 45 ± 4 *vs.* 41 ± 11 % for type I and II, respectively. Although no differences were observed in ACSA, mean FCSA was 25 % lower in smokers than in non-smokers (P=0.001; Figure 6.1E). Although ~50% lower in the smokers, capillarisation in terms of LCFR for both fibre types and overall C/F ratio did not differ between smokers and non-smokers, with 1.07 ± 0.23 *vs.* 1.53 ± 0.35 for smokers and non-smokers, respectively (Figure 6.1F), but was higher in type I than type II fibres (P<0.001). The CFD, however, was similar for type I and type II fibres and did not differ between smokers and non-smokers (365 ± 69 *vs.* 354 ± 95 for type I and 344 ± 63 *vs.* 289 ± 109 for type II fibres in non-smokers and smokers, respectively). Similarly, the overall capillary density did not differ significantly between smokers and non-smokers (314 ± 72 *vs.* 333 ± 60 mm^{-2}). The $log_R SD$ was also similar in both groups: 0.098 ± 0.009 *vs.* 0.108 ± 0.016 for non-smokers and smokers, respectively. SDH-activity was higher in type I than type II fibres (P<0.001). However, the SDH-activity (Figure 6.1G) and FCSA x SDH-activity (spatially integrated SDH-activity) of each fibre type were similar in the two groups, indicating a similar total oxidative capacity of the muscle cells in smokers and non-smokers. The myoglobin concentration was higher in type I than type II cells (P<0.001), but was not significantly altered by smoking (Figure 6.1H).

Interestingly, the correlation coefficients of the fatigue resistance and overall SDH-activity was 0.93 (P=0.02) in controls, but was not significant in smokers (r=-0.67; P=0.21; Figure 6.2). The slope of the relationship between SDH-activity and fatigue resistance was significantly different between smokers and non-smokers: 3.56 ± 0.8 x 10^6 *vs.* -2.43 ± 1.52 x 10^6 % s µm A_{660}^{-1} for non-smokers and smokers respectively (P=0.01).

The correlation of the percentage of type I fibres and the fatigue resistance in the non-smokers was 0.97 (P=0.01), while it was not significant in the smokers (r =-0.60, P=0.28).

No significant relationships were observed between the capillarisation or myoglobin concentration and fatigue resistance in either group. Neither were any parameters (percentage type I, SDH-activity, capillarisation, myoglobin) related to smoking history in terms of cigarettes d^{-1} or cigarette pack years (data not shown).

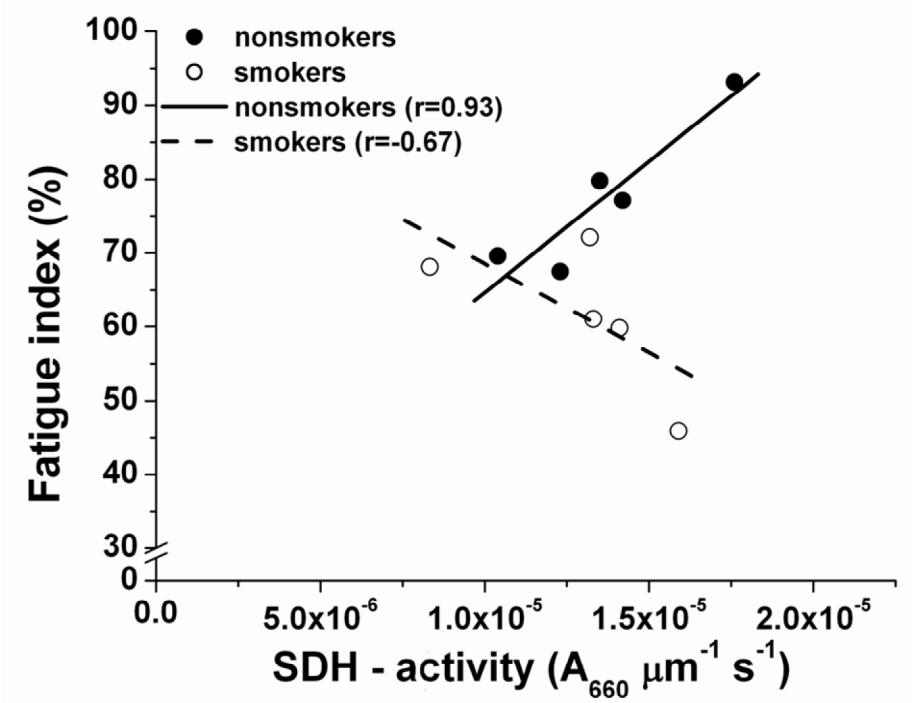

Figure 6.2. Effects of smoking on the relation between succinate dehydrogenase (SDH)-activity and fatigue index. The fatigue index is the torque at the end of the fatigue resistance test, expressed as a percentage of the initial torque. A linear relationship between SDH-activity and fatigue resistance was found for the non-smokers (P=0.02), but was absent in the smokers (P=0.21).

Discussion

The main observation of this study is that the lower fatigue resistance in smokers compared with non-smokers (Chapter 3) is not related to differences in fibre type composition, oxidative capacity, myoglobin concentration or capillarisation. The correlation between SDH-activity and fatigue resistance in non-smokers was lost in smokers. These data suggest that the reduced fatigue resistance in smokers is not due to alterations in determinants of oxidative metabolism, but may be related to factors in cigarette smoke that affect the delivery of oxygen and the function of the respiratory chain.

We took special care to match the physical activity level of the non-smokers and smokers. This is important as there is a positive correlation between physical activity and SDH-activity (Den Hoed *et al.*, 2008), which we also observed in our data (r=0.83). The absence of a significant relationship between myoglobin concentration or capillary density and fatigue resistance in the vastus lateralis muscle of smokers and non-smokers suggests that these parameters are not determinants of the fatigue resistance elicited by our protocol using electrical stimulation. Interestingly, the SDH-activity did not correlate with fatigue resistance in smokers (Figure 6.2).

Because of the small sample size there is a risk for false negative effects. We minimised this risk by selecting subjects for age, gender and physical activity level, and by using multilevel analysis. In addition, power analysis revealed that for a power of 0.8 for most of the parameters, except for LCFR, more than 50 subjects were required to reach significance for non-significant differences in figure 6.1, indicating that the differences between the means of these parameters are small anyway, and unlikely to explain the difference in fatigue resistance.

Cigarette smoking has been associated with an enhanced expression of factors involved in muscle protein degradation (Petersen *et al.*, 2007) and type I fibre atrophy (Montes de Oca *et al.*, 2008). Although the decline in ACSA was not significant, we observed that the FCSA of both type I and type II fibres in smokers was significantly smaller than in non-smokers. This reduction in FCSA would result in reduced oxygen diffusion distances from the periphery to the centre of the fibre (Hill, 1965) which may delay the development of fatigue. Reduced FCSA is associated with a lower minimal interstitial oxygen tension, which prevents an anoxic core in maximally working fibres (PO_{2crit}), calculated with Hill's oxygen diffusion model, including myoglobin-facilitated oxygen diffusion (for details, see Van Beek-Harmsen *et al.* 2004) in the type I and type II fibres in smokers than non-smokers, with 9.1 ± 1.8 *vs.* 6.8 ± 3.4 mm Hg in type I and 6.6 ± 1.7 *vs.* 4.3 ± 2.6 mm Hg in type II for non-smokers and smokers respectively. Despite this (non-significant) 25 to 34% reduction in PO_{2crit} at the level of the muscle fibre, overall fatigue resistance was lower in the smokers.

Taken together, the results of this study suggest that other factors determining oxygen supply and/or the ability to utilise oxygen are affected by smoking and limit muscle performance in smokers. A diminished oxygen supply may also occur when COHb is formed; the inhalation of CO until COHb reached 6% caused an 8% reduction in fatigue resistance (Morse *et al.*, 2008). Because the PO_{2crit} is reduced and the mean blood COHb level in smokers is reported to vary from 4 – 5 % (Hampson *et al.*, 2006), less than half of the difference in the fatigue index between smokers and non-smokers can be explained by this effect of CO. However, CO also causes a left-shift of the HbO_2 dissociation curve, hampering the release of oxygen, and it impairs the facilitated transport of oxygen within the muscle cell by binding to myoglobin (Gorman *et al.*, 2003). Finally, CO inhibits the function of complex IV of the electron transport chain (Alonso *et al.*, 2003). A combination of these factors attenuates not only delivery but also utilisation of oxygen. The delivery of oxygen may further be hampered by an impaired peripheral blood flow in smokers (Ronnemaa *et al.*, 1999), due to endothelial dysfunction through oxidative stress and the reduced bioavailability of nitric oxide (Ambrose & Barua, 2004; Montes de Oca *et al.*, 2008). The nature and relative contribution of each of these factors in determining smoking-related fatigue warrant further investigation.

We conclude that while muscle fibre size was smaller in the smokers, fibre type distribution, capillarisation and SDH-activity of the muscle are similar in physical activity-matched smokers and non-smokers. We suggest that the main cause of increased peripheral fatigue may be impaired oxygen delivery and/or utilisation by the muscle through acute effects of smoking, such as those conveyed by CO in cigarette smoke.

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CHAPTER 7

REGION-SPECIFIC ADAPTATIONS IN DETERMINANTS OF RAT SKELETAL MUSCLE OXYGENATION TO CHRONIC HYPOXIA

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Abstract

Chronic exposure to hypoxia is generally associated with muscle atrophy (i.e. reduction in muscle fiber cross-sectional area), reduced oxidative capacity and capillary growth. As some controversy still exists in the literature to what extend these changes are muscle and fiber type specific, we hypothesize that different regions of the same muscle would also respond differently to severe chronic hypoxia. To investigate this we compared the deep (oxidative) and superficial (glycolytic) region of the plantaris muscle of 8 male rats exposed to 4 weeks hypobaric hypoxia (410 mm Hg) with those of 9 normoxic rats. The haematocrit was higher in chronic hypoxic than control rats (59 vs. 50 %; $P<0.001$). Using histochemistry, we observed a 10% fibre atrophy ($P<0.05$) in both regions of the muscle, but no shift in fibre type composition and myoglobin concentration of the fibres. In hypoxic rats, the succinate dehydrogenase (SDH)-activity was elevated in fibres of each type in the superficial (25 %, $P<0.05$), but not in the deep region, while in the deep, but not the superficial region, the number of capillaries supplying a fibre was elevated (14 %, $P<0.05$). Model calculations showed that the region-specific alterations in fibre size, SDH-activity and capillary supply to a fibre prevented the occurrence of anoxic areas in the deep region, but not in the superficial region. We conclude that the determinants of tissue oxygenation show region specific adaptations, resulting in a marked differential effect on tissue oxygen tension.

Introduction

Chronic hypoxia has been suggested to be one of the factors that contribute to changes in muscle structure and function in patients suffering from chronic respiratory and cardiovascular diseases (Serres *et al.*, 1998; Gosker *et al.*, 2000; Raguso *et al.*, 2004; Chapter 1). Oxygen is crucial for aerobic energy metabolism and even a brief reduction in oxygen supply due to hypoxia leads to an acutely diminished muscle performance (Hogan *et al.*, 1999; Degens *et al.*, 2006b; Romer *et al.*, 2007). During long-term exposure, skeletal muscle adapts to the reduced oxygen tension by marked changes in skeletal muscle fibre size and cellular metabolism (Green *et al.*, 1989a; Ferretti *et al.*, 1990; Hoppeler & Vogt, 2001; Mathieu-Costello, 2001; Yun *et al.*, 2005).

An increase in capillary density in rat muscle after 6 weeks of exposure to 12% oxygen (Deveci *et al.*, 2001, 2002) may serve to facilitate oxygen supply to the muscle cells by reducing the diffusion distances of oxygen in the muscle tissue. This response is realised by angiogenesis (Deveci *et al.*, 2001, 2002) and/or a decrease in fibre cross-sectional area (FCSA, Banchero, 1987; Ferretti *et al.*, 1990; Bigard *et al.*, 1991). These results, however, are not consistent as an unaltered FCSA (Snyder *et al.*, 1985; Deveci *et al.*, 2002) and/or capillarisation (Sillau, 1980; Abdelmalki *et al.*, 1996) in response to chronic hypoxia have also been reported.

A factor that is often overlooked, but may have a major impact on the tissue oxygenation is the distribution of the capillaries: a more homogeneous distribution of capillaries might enhance tissue oxygenation, while a strong heterogeneous distribution of capillaries may result in anoxic areas (Piiper & Scheid, 1991; Degens *et al.*, 2006a; Goldman *et al.*, 2006). Differences between and within muscles have been observed, where capillaries in oxidative muscles and muscle regions are less heterogeneously distributed than glycolytic areas and muscles (Egginton *et al.*, 1988; Degens *et al.*, 1992). Hypoxia may result in a more homogeneously distributed capillary network, enhancing the tissue oxygenation.

Besides adaptations in capillarisation, chronic hypoxia might also affect energy metabolism. In both human and rat muscle a decreased oxidative capacity has been

reported and is accompanied by an increased glycolytic capacity (Green *et al.*, 1989b; Hoppeler *et al.*, 1990; Howald *et al.*, 1990; MacDougall *et al.*, 1991; Abdelmalki *et al.*, 1996; De Palma *et al.*, 2007). Others, however, report that the oxidative capacity is unchanged (Leon-Velarde *et al.*, 1993) or even increased after exposure to hypoxia (Gimenez *et al.*, 1977; Hochachka *et al.*, 1983).

It is clear that different adaptations to chronic hypoxia are reported in the literature. The observation that adaptations to hypoxia are muscle-specific and even specific to the region within a muscle could explain these discrepancies (Sillau & Banchero, 1977; Snyder *et al.*, 1985; Deveci *et al.*, 2001; Faucher *et al.*, 2005; Esteva *et al.*, 2008; Panisello *et al.*, 2008). Part of the controversy might be related to the duration and severity of exposure to normobaric or hypobaric hypoxia, the addition of a training programme to the exposure to hypoxia and/or the study of different muscles. Indeed, it has been reported that slow and fast muscles respond differently to chronic exposure of hypoxia (Deveci *et al.*, 2001; Faucher *et al.*, 2005), but also to chronic stimulation (Badr *et al.*, 2003) or disuse (Stevens *et al.*, 2000). Even within one muscle with a non-uniform fibre type distribution, the capillary adaptation to hypoxia seems to depend on the region of the muscle, where the hypoxia-induced angiogenesis was greater in muscles with a overall higher oxidative capacity and regions within a muscle with a lower oxidative capacity (Deveci *et al.*, 2001).

To date, most studies have focussed only on one aspect of adaptation of skeletal muscle to hypoxia, such as alterations in either oxidative capacity (Howald *et al.*, 1990), capillarisation (Deveci *et al.*, 2001; Mathieu-Costello, 2001; Deveci *et al.*, 2002), fibre type composition (Bigard *et al.*, 1991) or myoglobin concentration (Reynafarje, 1962). Of course the overall ability to utilize oxygen of the muscle cell depends on the interplay between capillary supply, myoglobin concentration and oxidative capacity and while there may be no adaptation in one factor, the muscle may still adapt to ensure adequate tissue oxygenation by alterations in other factors. To investigate whether adaptations to chronic hypoxia were fibre type- and/or region-specific we used the rat plantaris muscle that contains distinct oxidative and glycolytic regions. Rats were exposed for 4 weeks to chronic hypobaric hypoxia (equivalent to an altitude of ~5000 m). We used an integrative approach to assess the skeletal muscle fibre adaptations to chronic hypoxia by determining changes in fibre

type, size, capillary supply, oxidative capacity and myoglobin concentration in individual muscle cells in serial histological sections. We hypothesised that (a) overall there is atrophy and a decrease in oxidative capacity, (b) in the superficial region of the muscle angiogenesis is more pronounced than in the deep region and (c) that these adaptations prevent the occurrence of anoxic areas during hypoxia in both regions of the muscle. To address the latter hypothesis and to obtain an indication whether possible adaptations are sufficient to prevent anoxic tissue areas, we used a Krogh (Krogh, 1919; Hoofd, 1995) and a Hill model (Hill, 1965; Des Tombe *et al.*, 2002) to calculate the critical capillary and interstitial oxygen tensions required to prevent anoxic core at the muscle fibre's maximal oxygen consumption ($\dot{V}O_{2max}$).

Methods

A more detailed description of the methods applied in this chapter are given in the appendix.

Animals

Seventeen male Wistar rats, aged 8 weeks at the start of the study, were used. The experimental rats (n=8) were housed in a hypobaric hypoxic chamber at a barometric pressure of 410 mm Hg and 20.9 % O_2 (equivalent to ~5000 m altitude). The normobaric rats (n=9) lived in the same room, but at ambient pressure (~760 mm Hg). All rats were kept at room temperature and a 12 h light-dark cycle. Food and water were given *ad libitum* and the cage was opened for cleaning maximal half an hour every week. After 4 weeks exposure to hypobaric hypoxia or the normobaric condition, the rats were anaesthetised by intraperitoneal injection of pentobarbital sodium (70 mg kg^{-1}) and the plantaris muscle was dissected from the surrounded tissue blotted dry and weighed. Then the muscle was pinned on cork in a slightly stretched condition to minimise any bias in the determination of fibre cross-sectional area and capillary parameters, related to differences in muscle length, quickly frozen in liquid nitrogen and stored at -80 °C until further analysis. All protocols and procedures were approved by the University of Nijmegen institutional animal care and use committee.

Haematocrit

The haematocrit was determined in blood samples taken from the right orbita.

Myosin heavy chain composition

To determine the myosin heavy chain (MyHC) composition (type I, IIA, IIX and IIB) of the plantaris muscle we used a modification of the SDS-PAGE method used previously (Degens *et al.,* 1998). In short, a 10 µm section was dissolved in 300 µl Laemmli buffer (Laemmli, 1970) and 11 µl was loaded onto 6 % acrylamide gels. Gels were run at 250 V for 25 hours and subsequently stained with a Silverstain Plus Kit (Biorad, Hemel Hempstead, UK).

Histological section staining

Serial 10 µm sections from the middle of the plantaris muscle were cut on a cryostat at -20 °C and mounted on polylysine-coated slides. The sections were stored at -80 °C until further analysis, unless otherwise stated. All sections were mounted in glycerin-gelatin after staining.

Myosin ATPase

Unfixed sections were stained for myosin ATPase to classify fibres as I, IIa and IIx/b after pre-incubation at pH 4.55, as described previously (Brooke & Kaiser, 1970; appendix). The fibre type distribution was given as the area percentage of each fibre type.

Succinate dehydrogenase (SDH) activity

The SDH-activity in individual muscle cells in histological sections was determined immediately after cutting as described previously (Pool *et al.*, 1979; appendix).

Myoglobin concentration

The myoglobin concentration in individual muscle cells was determined as described previously (Van Beek-Harmsen *et al.*, 2004; appendix).

Capillarisation

Capillaries were depicted by staining sections for alkaline phosphatase as described previously (Degens *et al.*, 1992; Deveci *et al.*, 2001, 2002; appendix). The

capillarisation was analysed using the method of capillary domains (Hoofd *et al.*, 1985; Degens *et al.*, 1992; appendix).

Modelling of skeletal muscle tissue oxygenation

We applied the Hill and an extended Krogh model to obtain some insight on how the observed adaptations affected tissue oxygenation during hypoxia. Both models assume that oxygen diffuses over the cell membrane and through the cytoplasm into the mitochondria and take into account myoglobin-facilitated oxygen diffusion. The Hill model assumes a homogeneous oxygen tension (PO_2) in the interstitial space around the fibre, while the Krogh model assumes that oxygen diffuses from point-sources of oxygen (capillaries). We assumed that at $\dot{V}O_{2max}$ the flow through and PO_2 in all capillaries is the same.

Hill model

The critical extra-cellular oxygen tension required to prevent the development of an anoxic core in a cylindrical cell (PO_{2crit} in mm Hg) at maximal rate of oxygen consumption of the muscle fibre ($\dot{V}O_{2max}$) was calculated with the Hill model (Hill, 1965; Murray, 1974):

$$PO_{2crit}=(\dot{V}O_{2max} \cdot FCSA - 4\pi \cdot D_{Mb} \cdot [MbO2]_R) / 4\pi \cdot \alpha_M \cdot DO_2$$

where $\dot{V}O_{2max}$ in mM s^{-1} is calculated using SDH-activity (Van der Laarse *et al.*, 1989), FCSA in mm^2, D_{Mb} is the radial diffusion coefficient of myoglobin (0.27×10^{-4} mm^2 s^{-1}, Baylor & Pape (1988)), $[MbO2]_R$ is the concentration of oxygenated myoglobin at the sacrolemma (in mM) and calculated using the methods described in Des Tombe *et al.* (2002), α_M is the solubility of oxygen in skeletal muscle, DO_2 is the diffusion coefficient for oxygen in skeletal muscle. The product of the latter two ($\alpha_M \cdot DO_2$) is known as the Krogh's diffusion coefficient (2.00 nM mm^2 mm Hg^{-1} s^{-1}, Van der Laarse *et al.*, 2005).

Krogh model

The critical oxygen tension at the capillary required to prevent the development of anoxic tissue areas when the muscle is working at $\dot{V}O_{2max}$ (PO_{2cap} in mm Hg) was calculated using a universal Krogh's tissue model (Hoofd, 1995). We used

photomicrographs of sections with a representative capillarisation for each region and condition. For the model calculations the capillary radius was set at 2.4 μm. The weighted average $\dot{V}O_{2max}$ and myoglobin concentration were fed into the model. Where the Hill model calculates a PO_2 that should be uniformly present around a muscle fibre, Krogh's tissue model calculates a minimal PO_2 at the capillary that ensures the absence of anoxic areas.

We also used the Krogh's tissue model to estimate the distribution of oxygen pressure within the muscle tissue during normoxia (PaO_2 100 mm Hg), acute hypoxia (40 mm Hg, both conditions using tissue characteristics of the normoxic situation) or chronic hypoxia (40 mm Hg, using the tissue characteristics of the tissue adapted to hypoxia).

Statistical analysis

Independent *t*-tests were used to compare means between the two groups. Multilevel analysis (MLwiN, version 2.02, Centre for Multilevel Modelling, London, UK) was used to test for significant differences in capillarity, oxidative capacity, myoglobin concentration and fibre size between region, fibre type and hypoxia/normoxia. Multilevel analysis can be considered as an extension to the commonly used linear regression analysis, which has the disadvantage that only a continuous outcome variable can be analysed (Twisk, 2006). Differences were considered significant at $P<0.05$. Values are presented as mean ± SEM.

Results

The body mass of the hypoxic (357 ± 12 g) and control (382 ± 8 g) rats were not significantly different. The mass of the hypoxic plantaris muscle was 7 % lower compared to the control, but was not significantly different (397 ± 23 *vs.* 370 ± 13 mg in control and hypoxia, respectively). The hypoxic rats had a higher haematocrit than the control rats: 50 ± 1 *vs.* 59 ± 1 % ($P<0.001$). In total, 990 control and 1130 hypoxic fibres were analysed. For each fibre all parameters were assessed in serial sections; a typical example is shown in Figure 7.1.

Fibre type composition and fibre cross sectional area (FCSA)

The overall myosin heavy chain composition of the plantaris muscle did not change significantly after exposure to hypoxia (Figure 7.2). The deep region had a higher proportion of type I fibres and a lower proportion of type IIx/b fibres than the superficial region ($P<0.001$), while there was no significant regional difference in the proportion of type IIa fibres. In line with the myosin heavy chain data, hypoxia did not result in an altered fibre type composition (Figure 7.3A).

In both regions, the size of the fibres was in the order I < IIa < IIx/b ($P<0.05$). Type I fibres in the deep region were larger and type IIx/b fibres smaller than those of the superficial region ($P<0.05$), while type IIa fibres had a similar size in both regions. Hypoxia resulted in an overall 10% reduction in FCSA ($P<0.05$; Figure 7.3B). The absence of an interaction between fibre type and/or region with hypoxia indicates that this effect was similar for each fibre type and independent of region.

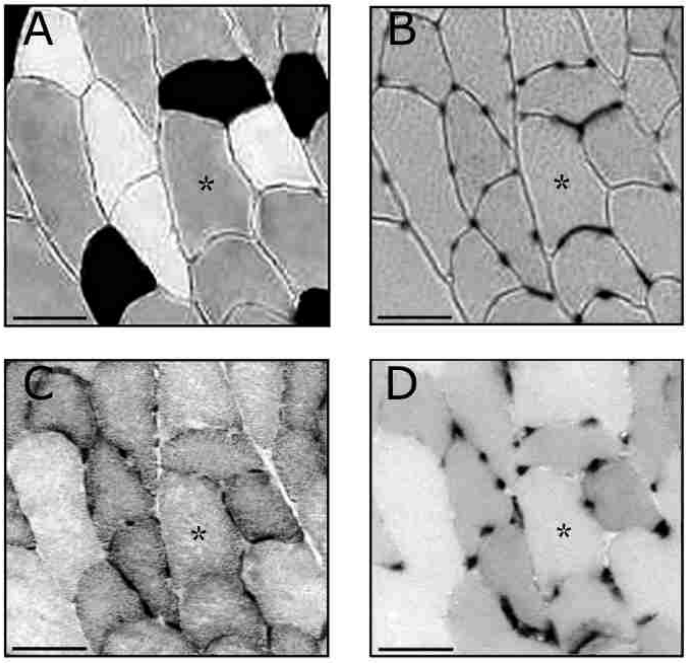

Figure 7.1. Typical example of serial sections stained for (**A**) ATPase after pre-incubation at pH 4.55, (**B**) capillaries, (**C**) succinate dehydrogenase (SDH) activity and (**D**) myoglobin concentration (note the dark spots stained for haemoglobin). Type I cells (stained dark for ATPase) had a higher capillary fibre density, higher SDH-activity and more myoglobin. * indicates same fibre in each section; bar presents 50 μm.

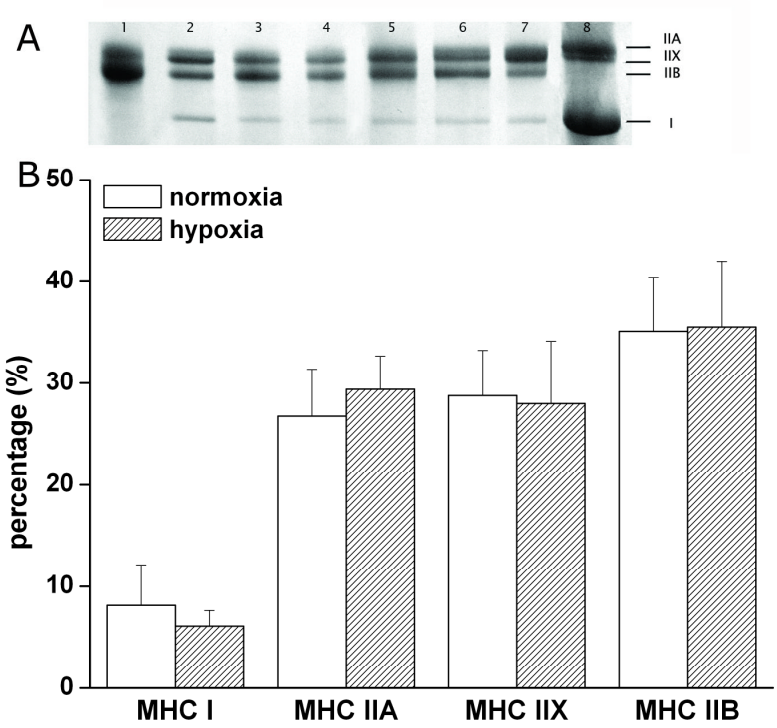

Figure 7.2. (**A**) Myosin heavy chain (MyHC) composition in the EDL (lane 1), normoxic plantaris muscle (lane 2-4), hypoxic plantaris muscle (lane 5-7) and soleus muscle (lane 8) of rats using 6% gel electrophoresis. (**B**) No significant differences were observed between normoxia (n=8) and hypoxia (n=9).

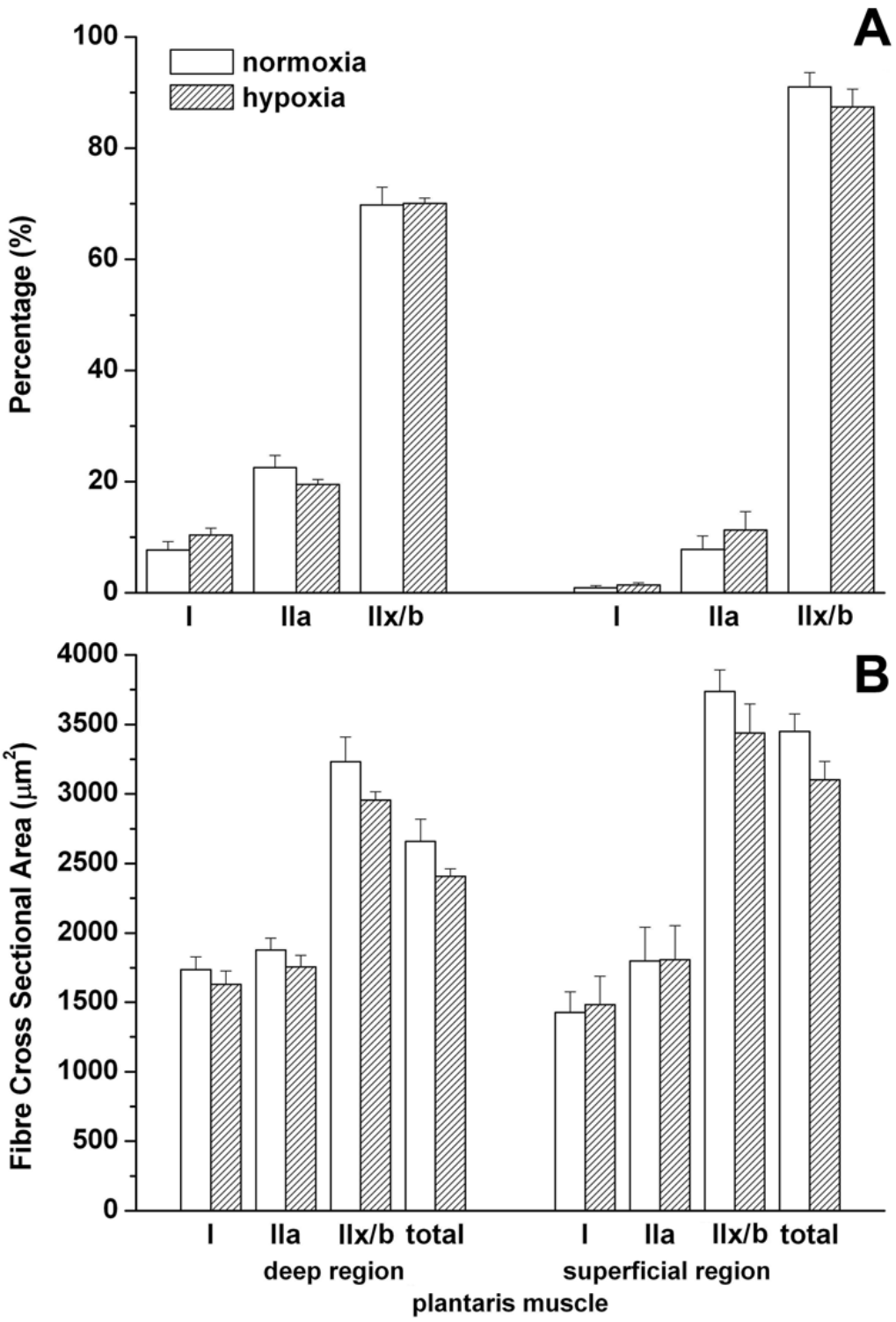

Figure 7.3. (**A**) Effects of hypoxia on the muscle fibre type distribution (percentage area occupied) and (**B**) fibre cross sectional area (FCSA) in the deep and superficial region of the plantaris muscle. The deep (oxidative) region has a significantly higher percentage of type I fibres ($P<0.001$) compared to the superficial (glycolytic) region. No significant fibre type shift was observed after 4 weeks of hypoxia. FCSA decreased significantly after exposure to hypoxia ($P<0.05$) in all fibre types and both regions. See *Results* section for further statistically significant differences between types and regions.

Succinate dehydrogenase (SDH) activity

SDH-activity in type IIx/b fibres was lower than that in the other fibres ($P<0.001$; Figure 7.4A). Interestingly, in the deep region only, the SDH-activity of type IIa fibres was higher than that of type I fibres ($P<0.001$). Overall, the SDH-activity was 26% lower in the superficial region than the deep region ($P<0.001$). This was explicable by the larger number of type IIx/b fibres in the superficial than the deep region. In addition, the SDH-activity in the type IIx/b fibres in the superficial region was lower than that in the deep region ($P<0.001$), while there was no significant difference in activity in the type I and IIa between the two regions. Hypoxia induced a 25% increase in the SDH-activity in fibres of each type in the superficial region ($P<0.05$), while no change in the SDH-activity occurred in the deep region.

The integrated SDH-activity is proportional to the $\dot{V}O_{2max}$ per mm fibre length (in nmol mm^{-1} s^{-1}) of the cell (Van der Laarse *et al.*, 1989). In the control rats, type I fibres had a lower integrated SDH-activity compared to type II fibres (IIa and IIx/b; $P<0.001$), while there was no difference between IIa and IIx/b fibres (Figure 7.4B). The integrated SDH-activity was higher in fibres from the deep compared to the superficial region ($P<0.001$). This was due to a higher integrated SDH-activity of type II ($P<0.001$), but not type I fibres, in the deep than the superficial region of the muscle. After exposure to hypoxia, this difference between the two regions disappeared ($P=1.00$)

Myoglobin concentration

Overall, fibres in the superficial region of the plantaris muscle had a 30% lower myoglobin concentration ([Mb]) than those in the deep region ($P<0.001$, Figure 7.5). The [Mb] of type IIa fibres in both the deep and superficial region was higher than that of type I fibres ($P<0.05$), while type IIx/b fibres had the lowest [Mb] ($P<0.001$). Hypoxia did not significantly affect [Mb] in fibres of all type or region.

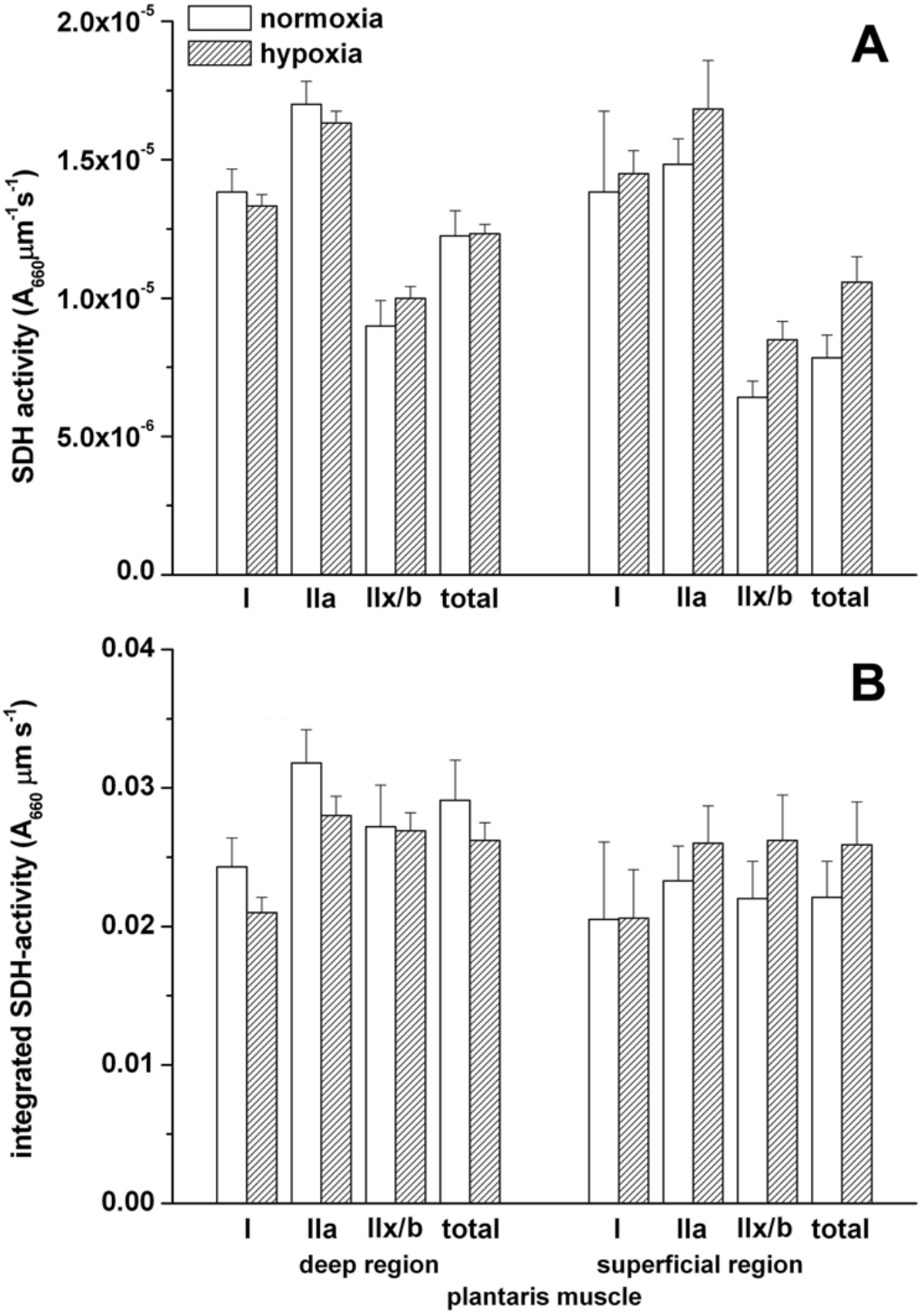

Figure 7.4. (**A**) Succinate dehydrogenase (SDH)-activity and (**B**) spatially integrated SDH-activity in different fibre types and regions of the plantaris muscle after 4 weeks hypoxia compared to normoxia. The SDH-activity in the superficial region was increased by 25% in the hypoxic group *(*$P<0.05$), but this did not occur in the deep region. The difference in spatially integrated SDH-activity between the deep and superficial region which was apparent in the control rats ($P<0.001$), disappeared after hypoxia ($P=1$). See *Results* section for further statistically significant differences between types and regions.

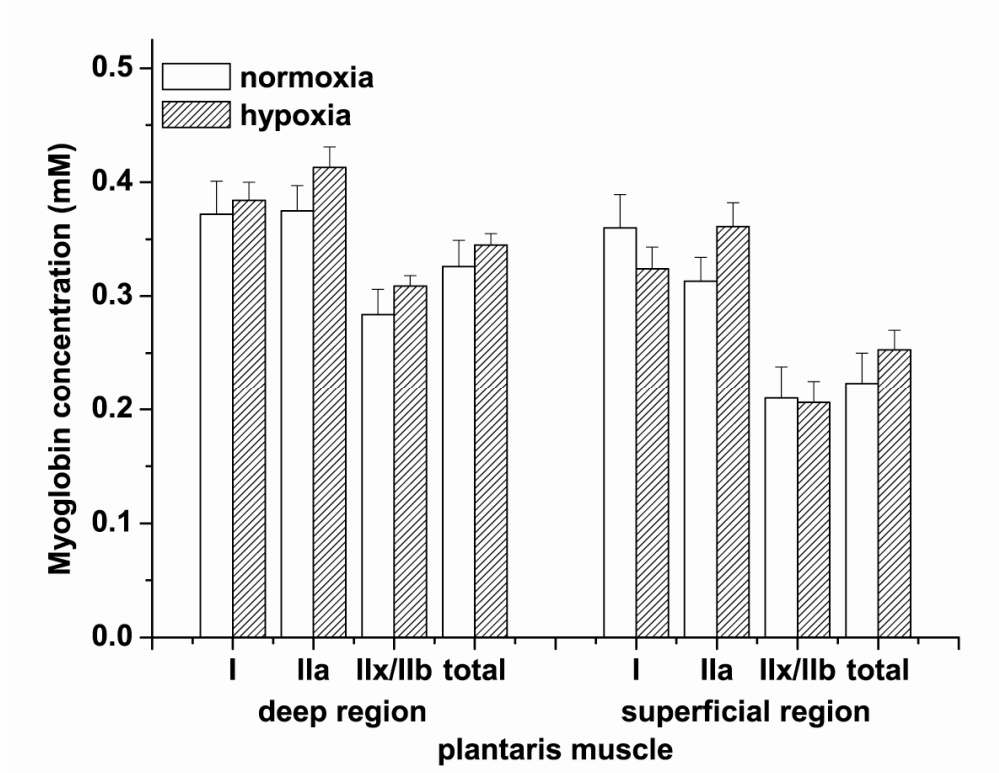

Figure 7.5. Myoglobin concentration in different fibre types and regions of the plantaris muscle after 4 weeks hypoxia compared to normoxia. No significant differences were observed after exposure to hypoxia. See *Results* section for statistically significant differences between fibre types and regions.

Capillarisation

The local capillary to fibre ratio (LCFR) for each fibre type and the overall capillary to fibre ratio (C/F, depicted as 'total') are displayed in Figure 7.6A. In both regions the LCFR of type IIx/b fibres was higher than that of the other fibres ($P<0.001$). In the deep region, type I and IIa fibres had a similar LCFR, while in the superficial region the LCFR of the type IIa was higher compared to type I fibres ($P<0.001$). The LCFR for each fibre type was higher in the deep compared to the superficial region ($P<0.001$). There was an interaction between region and hypoxia ($P<0.001$), which was apparent as a 14% increase in LCFR after hypoxia of fibres in the deep ($P<0.05$), but not in the superficial region.

The capillary fibre density (CFD) of fibres of each type was lower in the superficial than the deep region ($P<0.001$, Figure 7.6B). The CFD of type IIx/b was lower than that of type I and IIa fibres ($P<0.001$), which had a similar CFD. Hypoxia resulted in a 24% higher CFD in the deep ($P<0.01$), but not the superficial region of the muscles.

The heterogeneity of capillary spacing ($log_R SD$) was larger in the superficial compared to the deep (0.097 ± 0.013 *vs.* 0.112 ± 0.011) region ($P<0.001$), but was not significantly changed by hypoxia in either region (0.091 ± 0.010 and 0.118 ± 0.022; deep and superficial region, respectively).

Relationships between capillarisation, SDH-activity and FCSA

To investigate how the adaptations in response the hypoxia affected oxygen supply and demand, we calculated the relationships between LCFR, SDH-activity and FCSA for all fibre types. In Figure 7.7 the relations for type IIa and IIx/b fibres are shown, as they are the predominant fibres in the plantaris muscle.

As expected, LCFR was linearly related to the maximal oxygen uptake of the cell (as reflected by the spatially integrated SDH-activity) in type I (R^2 ranging from 0.165 - 0.640 for each region and condition, $P<0.001$), type IIa (R^2 ranging from 0.110 - 0.336, $P<0.001$, Figure 7.7A) and in type IIx/b (R^2 ranging from 0.070 - 0.284, $P<0.001$, Figure 7.7B), except for type IIx/b fibres in the normoxic superficial region ($R^2=0.012$, $P=0.06$). After hypoxia, the correlation coefficient and slope of this relation increased for the type IIx/b fibres in both regions ($P<0.001$). There was no significant relationship between LCFR and SDH-activity for the type I (R^2 ranging from 0.000 - 0.144) and IIa fibres (R^2 ranging from 0.008 - 0.052, Figure 7.7C) and in the type IIx/b fibres in the superficial region a slight negative relationship was observed ($R^2=0.018$, $P<0.01$, Figure 7.7D dotted line), which disappeared ($P<0.03$) after exposure to hypoxia ($R^2=0.000$).

While no correlation was found between LCFR and the SDH-activity, LCFR did positively correlate with FCSA in type I (R^2 ranging from 0.226 – 0.487), IIa (R^2 ranging from 0.115 – 0.491, Figure 7.7E) and IIx/b fibres (R^2 ranging from 0.127 – 0.341, Figure 7.7F). For all fibre types, the slope between FCSA and LCFR significantly increased in the deep ($P<0.05$), but not in the superficial region after hypoxia. Interestingly, while the region-specific difference in the slope was not significant in normoxia, hypoxia amplified the difference in the slope between the deep and superficial region for all fibre types, which was entirely due to an increased slope in the deep region only. This indicated that at a given FCSA the type I, IIa and IIx/b fibres in the superficial region had a similar LCFR, while those in the deep region had a higher LCFR after hypoxia ($P<0.001$). This was reflected by the increased CFD for each fibre type in the deep, but not in the superficial region during

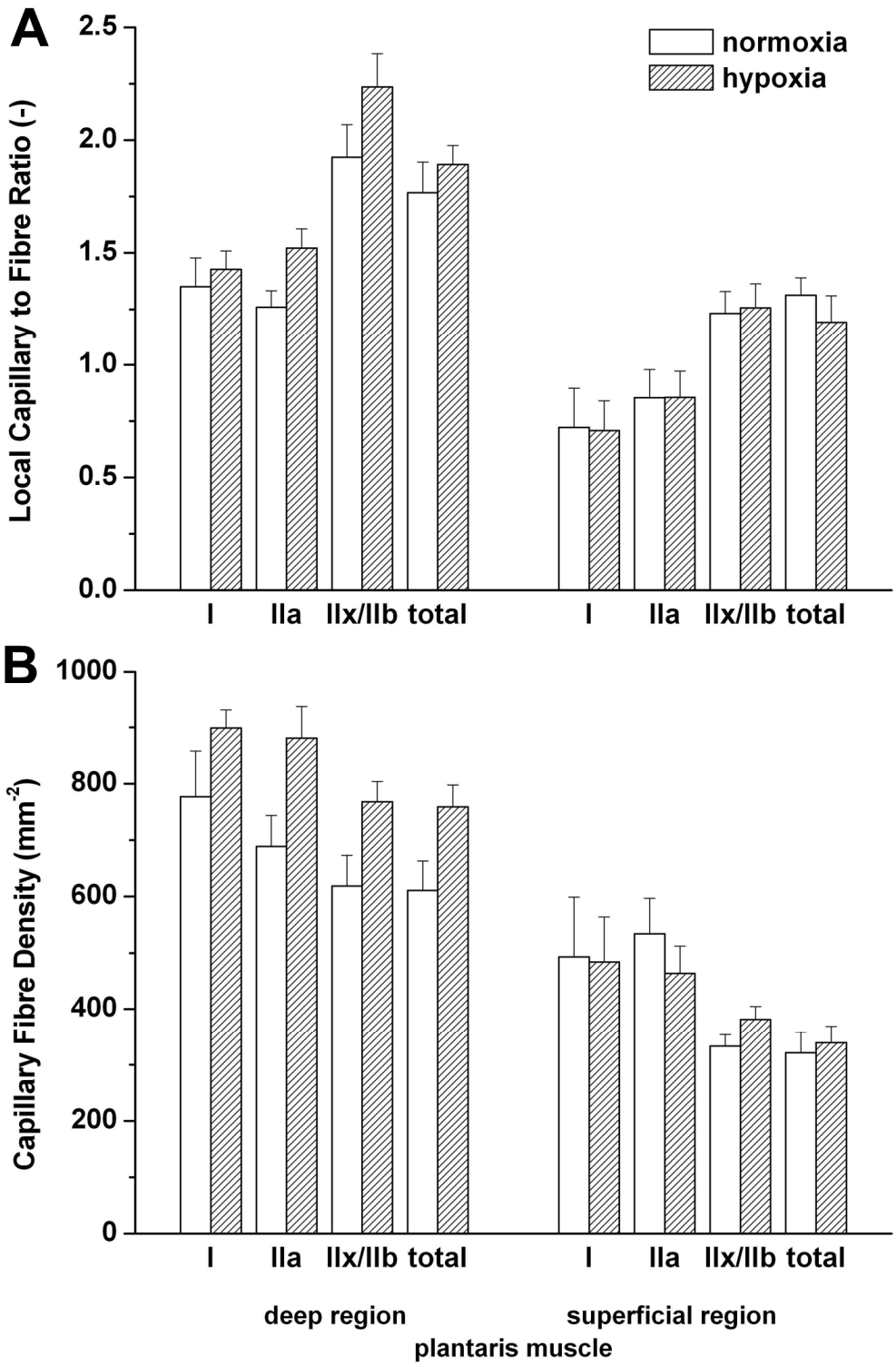

Figure 7.6. (**A**) Local capillary to fibre ratio (LCFR) and (**B**) capillary fibre density (CFD) in different fibre types and regions of the plantaris muscle after 4 weeks hypoxia compared to control values. Note that the total value in both regions is the overall capillary to fibre ratio (C/F) (**A**) or overall capillary density (CD) (**B**). Hypoxia increased LCFR in the deep region ($P<0.05$), but not in the superficial region. CFD increased in the deep ($P<0.01$), but not in the superficial region. See Results section for further statistically significant differences between types and regions.

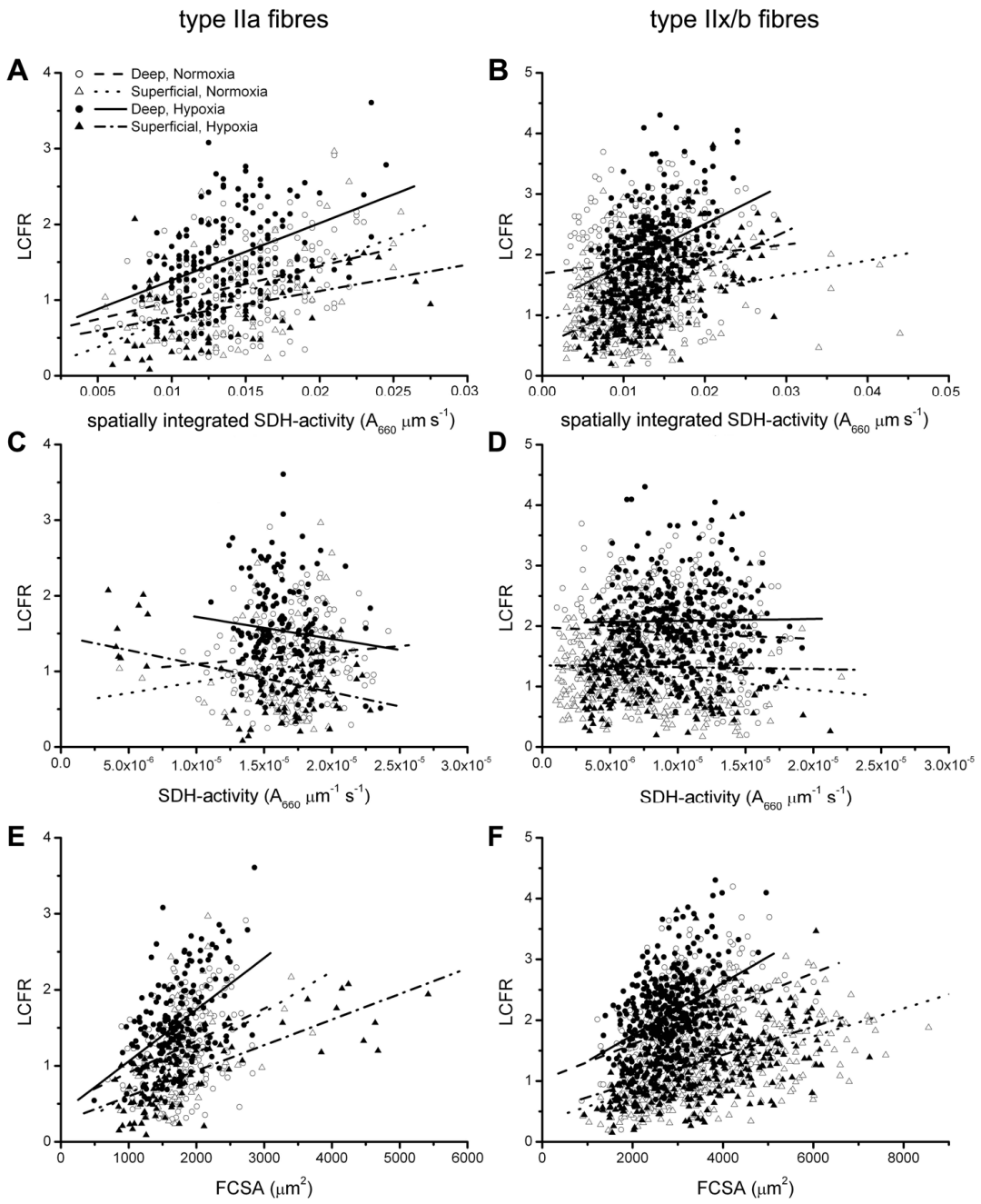

Figure 7.7. Relationship between LCFR and spatially integrated SDH-activity (**A** and **B**), SDH-activity (**C** and **D**) and FCSA (**E** and **F**) for type IIa (**A**, **C** and **E**) and IIx/b fibres (**B**, **D** and **F**). See legend for the conditions and regions. The correlation between the spatially integrated SDH-activity and the capillary supply (**A** and **B**) was due to a significant correlation between FCSA and LCFR (**E** and **F**), rather than a correlation between LCFR and SDH-activity (**C** and **D**). For R^2 and P-values, see Results section.

hypoxia (Figure 7.6B). Overall, the CFD of type I fibres was higher than that for type IIa fibres. Type IIx/b fibres had the lowest CFD ($P<0.001$).

Model calculations

Model calculations were used to asses the functional consequences of the observed adaptations. During normoxia the PO_{2crit} for each individual muscle fibre (Hill model) was higher in the deep compared to the superficial region ($P<0.001$; Figure 7.8A). In response to chronic hypoxia, the regional difference in PO_{2crit} disappeared, due to a decrease in the deep and an increase in the superficial region (interaction between hypoxia and region, $P<0.001$). The decrease in PO_{2crit} in the deep region was caused by muscle fibre atrophy, while the increase in the superficial region resulted from an increase in SDH-activity.

Subsequently, we calculated the PO_2 that is required in the capillaries to prevent the occurrence of any anoxic area at $\dot{V}O_{2max}$ (as was calculated for PO_{2crit}), which is referred to as the critical capillary PO_2 or PO_{2cap}. Thereto, we chose photographs with representative capillary distributions for each region and condition. The PO_{2cap} in the superficial region was higher compared to the deep region. The PO_{2cap} in the deep region did not differ between normoxia and hypoxia, but hypoxia resulted in a 25% increase in PO_{2cap} in the superficial region of the muscle (Figure 7.8B).

The PO_2 distribution, shown as a cumulative curve (in %), in the muscle tissue at $\dot{V}O_{2max}$ was calculated using the Krogh model in normoxia (Figure 7.9A capillary PO_2 set at 100 mm Hg), acute hypoxia (Figure 7.9B capillary PO_2 set at 40 mm Hg) and during hypoxia (capillary PO_2 set at 40 mm Hg) in the adapted situation (Figure 7.9B). While during normoxia no anoxic regions were present in either the deep or superficial region of the muscle (Figure 7.9A), acute hypoxia (40 mm Hg) resulted in a marked left-shift in the PO_2 distribution in the tissue; during acute hypoxia approximately 10% of the skeletal muscle tissue in the superficial region was anoxic, while hardly any anoxic regions were observed in the deep region. The adaptations of the skeletal muscle tissue to chronic hypoxia resulted in a marked region-specific adaptation: while in the deep region a right-shift occurred compared to the acute hypoxic situation, indicating an improved tissue oxygenation, an unexpected left-shift was observed in the superficial region.

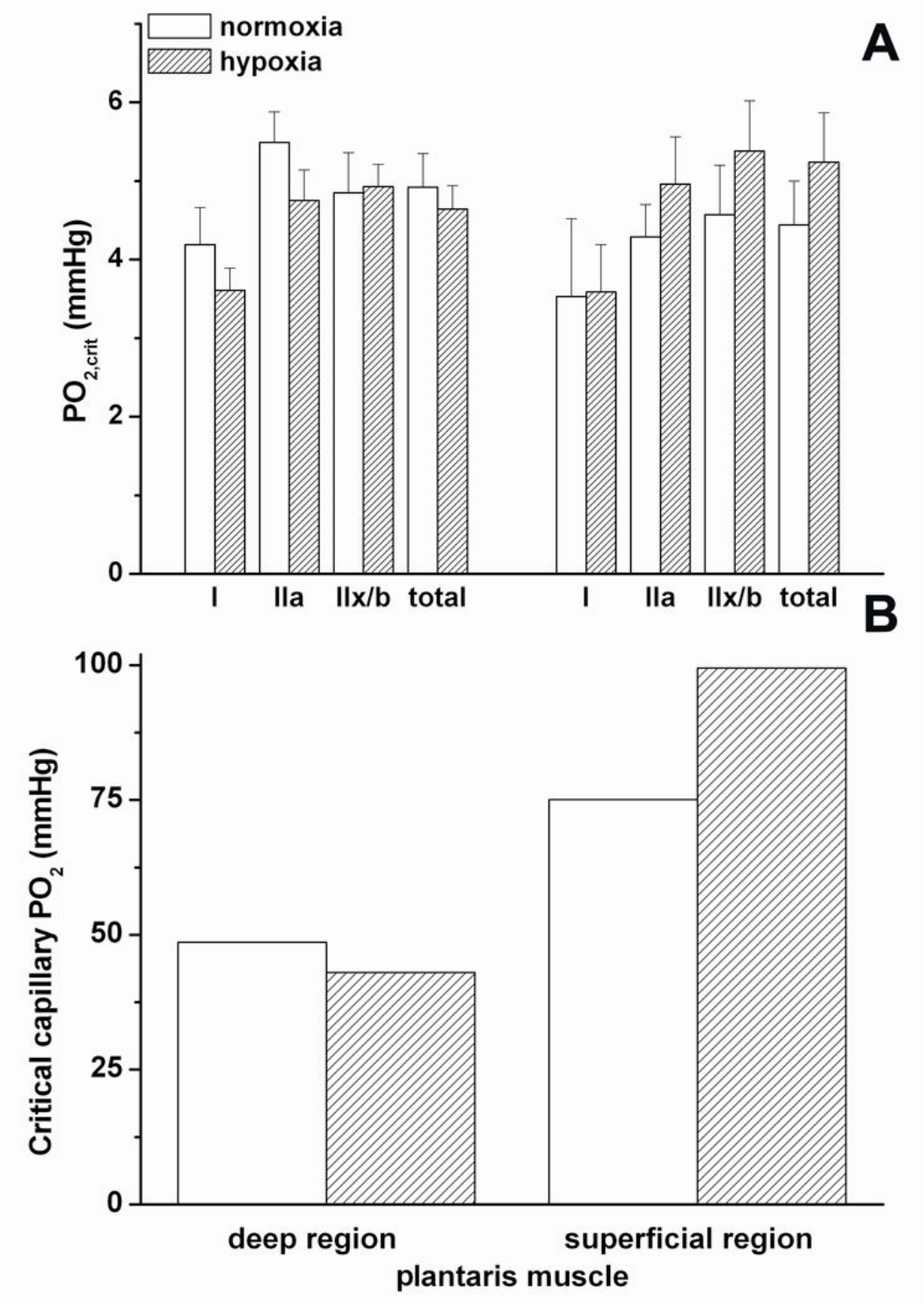

Figure 7.8. Minimal oxygen tension around the fibre (Hill model, **A**) and in the capillary (Krogh model, **B**) that prevents the development of an anoxic core when the fibres work at their maximal oxygen consumption ($\dot{V}O_{2max}$). The region-specific difference in PO_{2crit} in (A) disappeared after exposure to hypoxia. Using the Krogh-model (incorporating the capillary supply), the critical capillary PO_2 did not change in the deep region, while it increased with 25% in the superficial region.

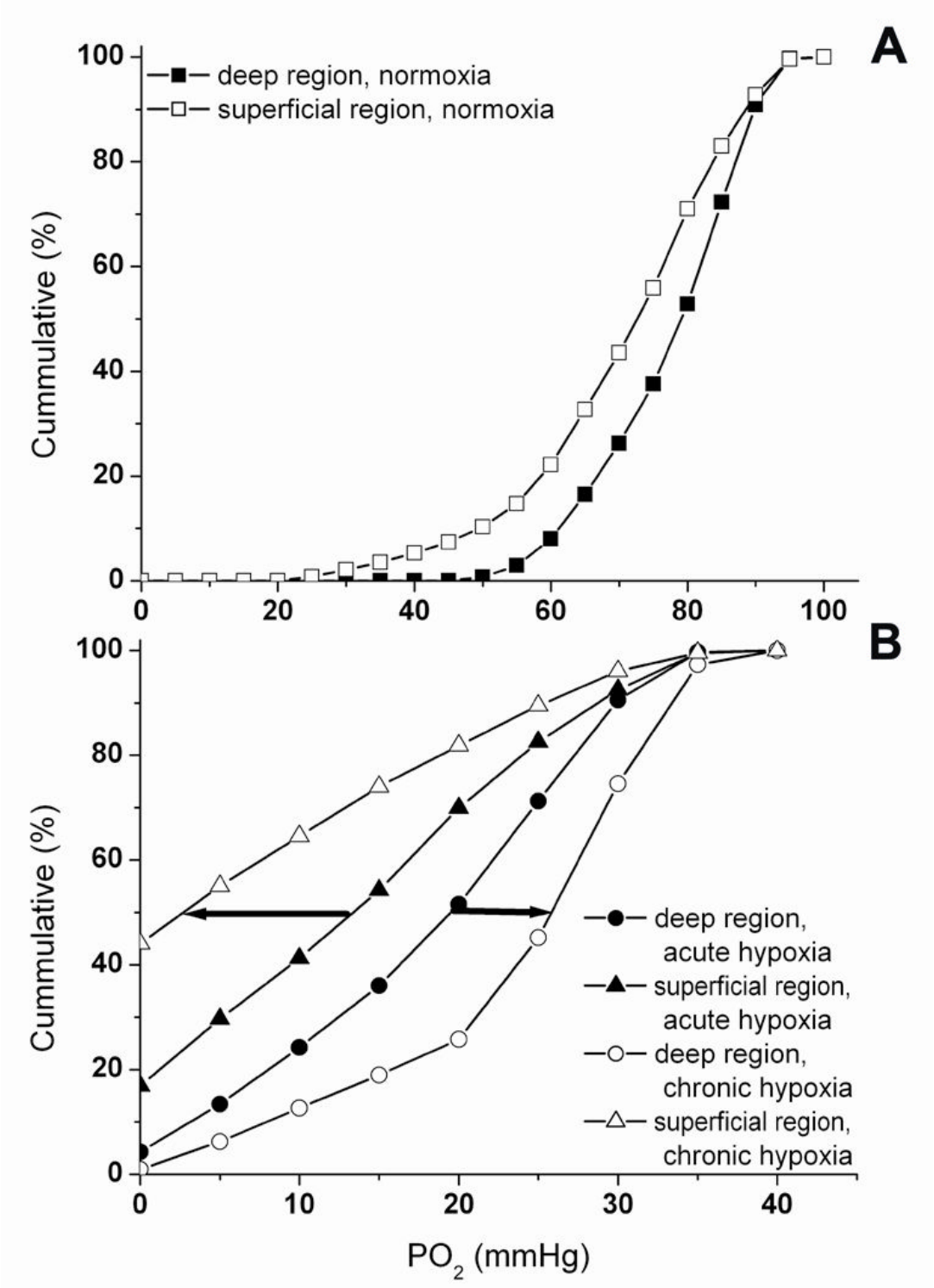

Figure 7.9. The cumulative PO_2 distribution (in %) in the muscle tissue calculated at $\dot{V}O_{2max}$ using the Krogh model in normoxia (**A**, PaO_2=100 mm Hg), during acute hypoxia (**B**: ● and ▲, PaO_2=40 mm Hg) and after adaptations to chronic hypoxia (**B**: ○ and Δ). A marked differential response in both regions can be observed.

Discussion

Using an integrative approach to study the parameters determining the oxygen delivery and utilization, we observed that the muscular adaptations to chronic hypoxia are region-specific and not fibre type-specific, which was in accordance with our hypothesis. Overall, hypoxia did cause a ~10% muscle fibre atrophy, irrespective of fibre type and muscle region. In the deep oxidative region of the plantaris muscle this and angiogenesis resulted in improved capillarisation. In the superficial glycolytic region there was no angiogenesis, but contrary to our expectations, a 25% increase in the oxidative capacity, indicating mitochondrial biogenesis. The absence of a change in the oxidative capacity in the deep (oxidative) region of the muscle indicates that the loss of mitochondria was proportional to the degree of atrophy. While the adaptations to chronic hypoxia in the deep region attenuated the reduction in tissue PO_2 and prevented the occurrence of anoxic areas during hypoxia, the increased oxidative capacity in the superficial region appeared to aggravate problems with tissue oxygenation during conditions of elevated oxygen demand by the muscle.

What determines the capillary supply in skeletal muscle in normoxia and hypoxia?

The general idea is that in many species, including birds (Mathieu-Costello *et al.*, 1992), humans (Bekedam *et al.*, 2003) and rats (this Chapter; Chapter 5) the capillary supply to a fibre is related to its oxidative capacity. This relationship is modulated by the metabolic surrounding of a fibre, where a fibre in an oxidative region has a larger capillary supply than a similar fibre surrounded by glycolytic fibres (Degens *et al.*, 1992). Here we show using quantitative histochemistry that although the number of capillaries supplying a fibre (LCFR) was indeed related to its $\dot{V}O_{2max}$, as estimated from its integrated SDH-activity, LCFR correlated more with fibre size (Figure 7.7, Maxwell *et al.*, 1980; Chapter 5). The lack of a correlation between SDH-activity per mm^{-2} and LCFR could be due, at least in part, to the fact that the mitochondrial density within a fibre decreases from the periphery to the centre of the fibre (Swatland, 1984; Chapter 5). We observed that the capillary supply per unit fibre perimeter is more or less constant (data not show; Degens *et al.*, 2006a;), suggesting that diffusion distance or capillary contact per fibre perimeter, rather than the total

number of mitochondria in a cell, is a more important determinant of the capillary supply to a fibre.

Capillaries do not only supply oxygen, but also remove waste products and heat from the exercising muscle cell. This may explain the absence of a positive relationship between the integrated SDH-activity and LCFR of the glycolytic fibres with a low oxidative capacity (type IIx/b) in the glycolytic region of the muscle (Figure 7B).

During exposure to hypoxia the integrated SDH-activity in these fibres increased and the relationship between spatially integrated SDH-activity and LCFR became stronger for the type IIx/b fibres only. This suggests that during hypoxia the function of the capillaries to deliver oxygen to the (previously low oxidative) muscle cells becomes more important.

Overall adaptations to chronic hypoxia

Similar to previous studies, we observed no differences in the fibre type composition (Green *et al.*, 1989b; Panisello *et al.*, 2008). Hypoxia did, however, cause a ~10% muscle atrophy (Ferretti *et al.*, 1990; Bigard *et al.*, 1991; Desplanches *et al.*, 1996; Deveci *et al.*, 2001) in fibres of each type and region, which may serve to decrease the diffusion distance from the capillaries to the interior of the muscle fibres.

The heterogeneity of capillary spacing ($\log_R SD$) may have a significant impact on tissue oxygenation. A homogeneous capillary spacing would improve tissue oxygenation (Piiper & Scheid, 1991; Goldman *et al.*, 2006; Degens *et al.*, 2006a In the deep oxidative region of the muscle, with a higher demand of oxygen, the capillaries were more homogenously distributed than in the superficial glycolytic region. Yet, we did not observe a more homogeneous distribution of capillaries after exposure to hypoxia, not even in the glycolytic region that became more oxidative. Also during hypertrophy (Degens *et al.*, 2009) and atrophy (Degens *et al.*, 2008) the heterogeneity of capillary spacing remains unaltered, and it has been suggested that the absence of changes might be related to physical constraints, determined by the morphology of the muscle fibres, of placing new capillaries (Degens *et al.*, 2009).

Myoglobin might act to facilitate diffusion of oxygen (Scholander, 1960). An elevated myoglobin concentration during hypoxia (Reynafarje, 1962) might thus serve to enhance intracellular oxygen transport and ensure an adequate tissue oxygenation. In agreement with previous observations (Van der Laarse *et al.*, 1985), we observed that oxidative type I and IIa fibres contain more myoglobin than

glycolytic IIx/b fibres (Van Beek-Harmsen et al., 2004). Although the oxidative capacity of fibres in the superficial region was elevated after hypoxia, we observed no change in the myoglobin concentration in fibres of any type or region after exposure to hypoxia. Other studies also report no change in the myoglobin protein content (Sillau & Banchero, 1977), even when endurance training was superimposed (Masuda *et al.*, 2001). Iron deficiency in the diet may limit the formation of functional myoglobin as it has been shown that elevated myoglobin mRNA levels do not necessarily translate into elevated myoglobin protein levels (Robach *et al.*, 2007). Iron deficiency, however, would also hamper mitochondrial synthesis (Hood *et al.*, 1992), something that did not appear to be limited in our study. Whatever the cause of the unchanged myoglobin content, our model calculations show that the facilitation of oxygen diffusion by myoglobin in our tissue was at most equivalent to a reduction of 5 mm Hg in the required PO_{2cap} (data not shown).

Region-specific adaptation to hypoxia

We observed that the deep oxidative and superficial glycolytic region of the muscle differed in their adaptations in capillarisation and oxidative capacity to chronic hypoxia. While in the deep region capillary proliferation took place, as reflected by the increased LCFR, no capillary proliferation occurred in the superficial region. On the other hand, the spatially integrated SDH-activity, reflecting the total number of mitochondria per fibre, decreased in the deep and increased in the superficial region. Our model calculations showed that the improved capillarisation in the deep region served to improve tissue oxygenation, while in the superficial region the adaptations did not appear to improve tissue oxygenation, as reflected by both an increased proportion of anoxic tissue areas and an elevated PO_{2crit}.

In the deep region the decreased integrated SDH-activity per fibre is explicable by atrophy. This, together with an unaltered SDH-activity per mm^2, reflecting the number of mitochondria per volume of tissue (Takekura & Yoshioka, 1989), indicates that atrophy is accompanied by a proportional loss of mitochondria in the deep region. The increased integrated SDH-activity in the superficial region, on the other hand, indicates that though atrophy also occurred in this region, mitochondrial biogenesis was elevated.

It is interesting to note that the adaptations were region- and not fibre type-specific. In other words, no matter what type a fibre was, its integrated SDH-activity was increased in the superficial and its capillary supply in the deep region. This is surprising as training appears to result in a fibre type-specific change in oxidative capacity (Takekura & Yoshioka, 1989). During training, however, only a subset of fibres may be recruited, while during hypoxia all fibres are affected. Nevertheless, though speculative, it might be that paracrine factors, such as VEGF, and/or other substances are excreted by or diffuse out of the cell in relatively larger quantities from cells that are recruited more, such as in the deep region (Clanton & Klawitter, 2001) than elsewhere, and hence affect the adaptation processes of fibres in different regions differently, overwhelming the fibre type-specific adaptations.

Possible explanation for differences in regional adaptation to hypoxia

Region-specific increase in capillarity

Similar to us, other investigators have also observed region-specifc adaptations in capillarisation (Deveci *et al.*, 2001, 2002; Badr *et al.*, 2003). During submaximal exercise particularly the deep oxidative region of the muscle is recruited (Kernell, 1998). To deliver the same amount of oxygen the blood flow to the muscle at a given submaximal workload is increased as is the extraction of oxygen (King *et al.*, 1987) and it is thus expected that particularly in the deep, and less so in the superficial glycolytic region, the endothelium is exposed to elevated shear stress, which is a potent stimulant for angiogenesis (Hudlicka *et al.*, 1992). Also in the superficial region an increased blood flow and shear stress is to be expected, which may, however, be too little to induce angiogenesis; if so it would suggest that there is a threshold in duration and/or magnitude of elevated blood flow, above which shear-stress would induce angiogenesis.

Region-specific adaptation in oxidative capacity

During hypoxia, even during submaximal exercise an accelerated breakdown of PCr and fall in pH occur (Hogan *et al.*, 1999). The increased rate of accumulation of metabolites may require the earlier recruitment of glycolytic fibres, even during submaximal activity. This more frequent recruitment may be compared to an endurance training programme inducing an increase in the oxidative capacity. An additional 5 % duration of recruitment has been shown to be sufficient to increase the

oxidative capacity of a muscle cell as seen with electrical stimulation (Kernell *et al.*, 1987) and also training during hypoxia induces an increase in the oxidative capacity (Terrados *et al.*, 1990; Abdelmalki *et al.*, 1996). It is not clear why the oxidative capacity in the deep region is not affected, but our observations are analogous to the observation that the increase in oxidative capacity following electrical stimulation is inversely related to the original oxidative capacity of the muscle or muscle region (Reichmann *et al.*, 1985).

The Hill model indicated that in the superficial region the PO_2 allowing the muscle cell to work at $\dot{V}O_{2max}$ was elevated during hypoxia. This corresponded with an increase in the capillary PO_2, calculated with the Krogh model, required for the tissue to work at $\dot{V}O_{2max}$ without the development of anoxic tissue areas. The higher PO_{2cap} than PO_{2crit} can be ascribed to the different assumptions of both models (homogeneous PO_2 around the fibre in the Hill model *vs.* oxygen diffusing from point sources in the Krogh-model). The increased oxidative capacity in the superficial region after chronic hypoxia does not appear to help, but rather to aggravate the situation as reflected by the increased prevalence of anoxic tissue areas as estimated with the Krogh model. Thus in terms of tissue oxygenation this seems to be an inappropriate adaptation to hypoxia. The respiration of the mitochondria is a hyperbolic function of the PO_2 the mitochondria experience and when the PO_2 becomes too low the respiration of the mitochondria will decrease (Gnaiger *et al.*, 2000). Yet, the demand for ATP by the fibres in the superficial region may increase, because of a more frequent recruitment as discussed above. An increase in mitochondria may enhance the flux of oxygen and hence total respiration, even though each individual mitochondrion is not working maximally (Hochachka *et al.*, 1983). According to our model calculations there are virtually no anoxic areas during hypoxia in the deep region of the muscle, suggesting that the mitochondria may still be able to work near-maximally. It is thus not necessary to increase their number to enhance flux in this region of the muscle.

Potential mediators for the region-specific adaptation in oxidative capacity are factors that are involved in transcriptional regulation of mitochondrial genes, such as PPARγ and its ligand PGC-1α (Kramer *et al.*, 2006). However, there are no studies on region-specific expression after exposure to hypoxia.

Conclusions

In conclusion, the adaptation of skeletal muscle to hypoxia is region- rather than fibre-type specific. Overall, hypoxia results in muscle atrophy. In oxidative regions, hypoxia results in angiogenesis which helps to attenuate a reduction in tissue PO_2, while fibres in a more glycolytic region of the muscle increase their oxidative capacity. Though the latter may cause problems with tissue oxygenation, we hypothesise that the increased oxidative capacity may serve to ensure an adequate aerobic ATP generation during hypoxia by maintaining the flux of oxygen. It is currently unknown which cellular pathways underlie these region-specific adaptations to chronic hypoxia.

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CHAPTER 8

GENERAL DISCUSSION

The aim of this final chapter is to provide an overview of what the results described in this thesis have added to the literature in this area and to discuss their possible relevance for patients with COPD. Subsequently, suggestions for future studies will be given.

The main objective of the present thesis was to investigate the effects of smoking and hypoxia on skeletal muscle structure and function. Smoking is the main cause of COPD, a condition where muscle weakness and, probably, premature fatigue seriously exacerbate the problems associated with the primary lung disease. Chronic systemic hypoxaemia is commonly observed in these patients and it is thus likely that this factor can contribute to muscle wasting and dysfunction.

The first three experimental chapters report the results of studies looking for changes in muscle function in subjects who were smokers but did not have any symptoms of lung disease. There were no obvious difference in strength or muscle sizes between the smokers and matched control subjects. A difference was detected, however, in fatigability but because there appeared to be sex differences it was necessary to examine this further to understand the basis of this difference.

In **Chapter 2, 3 and 4** we have used a fatigue test involving electrical evoked contractions of the quadriceps muscle (2 minutes 30 Hz with duty cycle of 0.5; 1 s on, 1 s off at an intensity equal to 30 % of MVC). Using such protocols, peripheral fatigue can be distinguished from central fatigue and contractile properties can be measured. This differs from more widely used methods that have used voluntary contractions to study muscle fatigue. Using voluntary contractions to assess skeletal muscle fatigue resistance does not allow one to distinguish between peripheral and central causes of fatigue. As electrical stimulation by-passes central motivation and neural causes of fatigue, protocols using electrically evoked contractions reflect only the contribution of factors beyond the motoneuron to muscle function and fatigue.

In Chapter **2** we report that, using this protocol, women were less prone to peripheral muscle fatigue than men, as already suggested by Hunter et al. (2006). We also observed slightly slower contractile properties in women than men suggesting that women may have a higher percentage of slower muscle fibres giving them an advantage in terms of aerobic metabolism or energy utilization. Several studies have

shown that women do have a higher proportion of type I fibres than men (Nygaard, 1981; Simoneau & Bouchard, 1989; Staron *et al.*, 2000; Jaworowski *et al.*, 2002; Roepstorff *et al.*, 2006), and the question arises what caused these differences. We have carefully selected our participants with regards to physical activity level, as a high level of physical activity is associated with a high fatigue resistance (Gerrits *et al.*, 2002), although probably by increasing aerobic capacity rather than changing MHC expression.

The fact that differences in fatigability between the sexes persisted even when the muscle was made ischaemic indicates that the difference probably lies in the rate of energy utilisation rather than energy supply, implicating differences in MHC content. One possibility that could be explored in future studies is whether, and to what extent, contractile properties are affected by differences in circulating hormones between men and women. That this might play a role is suggested by an increase in frog skeletal muscle fibre size and faster contractile properties (maximal shortening velocity, contraction and relaxation time) after testosterone supplementation (Girgenrath & Marsh, 2003). However, to what extent these findings can be extrapolated to humans and how circulating hormones affect fatigue resistance is currently unknown. While hypogonadism has been observed in male patients with COPD (Van Vliet *et al.*, 2005), possible changes in exercise intolerance and muscle structure and function after testosterone supplementation have not been extensively studied (Casaburi *et al.*, 2004). The few that are available indicate that testosterone supplementation in patients with COPD enhances muscle strength, while no data is currently available describing possible effects on contractile properties and muscle fatigue.

The results described in **Chapter 3** suggest that young men with a mild smoking history were less fatigue resistant than non-smoking, age- and physical activity-matched peers. The lower fatigue resistance was not related to the duration of the smoking when testing a cohort of smokers of a wide range of age and smoking history (**Chapter 4**) or with a lower oxidative capacity or changed fibre type (**Chapter 6**). Although the results from this last study need confirmation using a larger cohort of smokers, the results suggest that the higher muscle fatigability is not necessarily dose-related, but rather related to an acute, and possibly reversible, impairment either in the supply or ability to use oxygen for aerobic energy

generation by the muscle. In this respect the difference between smokers and non-smokers is not the same as between male and female control subjects discussed above. If the difference is due to altered oxygen supply or utilisation we would expect that smokers and non-smokers would have similar fatigue profiles when the muscles were made ischaemic. Unfortunately we were unable to test this hypothesis as the smokers were unwilling to undergo an ischaemic fatigue test (too painful) but this is clearly something that should be included in any future studies in this area.

CO is a major toxic component of cigarette smoke that causes a left-shift of the HbO_2 dissociation curve, hampering the release of oxygen. It also impairs the facilitated transport of oxygen within the muscle cell by myoglobin as its oxygen binding site is occupied by CO (Gorman *et al.*, 2003) and inhibits the function of complex IV of the electron transport chain (Alonso *et al.*, 2003). A combination of these factors attenuates not only delivery but also utilisation of oxygen. The delivery of oxygen may further be hampered by an impaired peripheral blood flow in smokers (Ronnemaa *et al.*, 1999), due to endothelial dysfunction through oxidative stress and the reduced bioavailability of nitric oxide (Montes de Oca *et al.*, 2008). A diminished oxygen supply may also occur when COHb is formed; the inhalation of CO until COHb reached 6 % caused an 8 % reduction in fatigue resistance (Morse *et al.*, 2008). The mean blood COHb level in smokers is reported to vary from 4 – 5 % (Hampson *et al.*, 2006), somewhat less than the levels used by Morse *et al.* (2008) suggesting that CO may not be the entire explanation for the increased fatigability. There are, however, other toxic substances in tobacco smoke, such as CN, that would have similar effects to CO.

Future work might focus on the effects of smoking cessation on muscle function and fatigue. If muscle fatigue is increased due to an acute impairment in oxygen delivery and/or utilization, then it is likely that the effect will be fairly rapidly reversed on smoking cessation. Earlier work on short-term smoking and smoking abstinence has already pointed in this direction. Smoking 3 cigarettes per hour for 5 hours has a detrimental effect on maximal oxygen uptake in humans (Hirsch *et al.*, 1985) and smoke inhalation acutely reduces swimming endurance in rats (Hrubes & Battig, 1970). A six or seven day abstinence, on the other hand, prolonged the time to exhaustion, mainly due to an improved oxygen transport to the muscles (Hashizume

et al., 2000). Also mitochondrial respiratory chain function (activity of complex IV), measured in peripheral lymphocytes, was lower in heavy smokers, but returned to normal values after 28 days of smoking abstinence (Cardellach *et al.*, 2003). However, to what degree skeletal muscle fatigue resistance will improve after smoking cessation is not known and a study to determine this would clearly be an important continuation of the present work.

Muscle fatigue protocols involving percutaneous electrical or magnetic stimulation are uncommon, but have been used successfully in patients with advanced COPD (Degens *et al.*, 2005; Swallow *et al.*, 2007). Using a comparable intensity and duty cycle to the present study, Swallow *et al.* (2007) observed that COPD patients experienced a higher muscle fatigability, while Degens *et al.* (2005) did not observe a difference in fatigue resistance between COPD patients and controls. The discrepancy in results might be related to the significantly lower physical activity levels in COPD patients than controls in the study by Swallow *et al.* (2007) while in the study by Degens *et al*. (2005) patients and control subjects were matched for physical activity level. It is interesting to note that all the COPD patients tested by Degens *et al.* (2005) had stopped smoking at the time of the experiment (H. Degens, personal communication) while it is not clear whether the patients in the Swallow study were smoking. Firm conclusions about the role of smoking in the exercise intolerance in patients with COPD cannot be drawn as long as smoking characteristics are not mentioned (or controlled).

The muscle atrophy and dysfunction seen in many patients with COPD is likely to have multiple causes including factors such as disuse, inflammation, reactive oxygen species and/or hypoxia. A small, but significant degree of muscle fibre atrophy was found in the small subgroup of smokers compared with non-smokers. Recently, it has been reported that smokers (without COPD) have a lower FCSA of type I fibres than non-smokers (Montes de Oca *et al.*, 2008), but it is not clear to what extent this result was due to an inappropriate selection of the control group (Wagner, 2008; Wüst *et al.*, 2008). The observation that the rate of protein synthesis in skeletal muscle is lower in smokers might be an explanation for the observed muscle atrophy (Petersen *et al.*, 2007).

The aim of the work described in **Chapter 7,** was to determine the effects of hypoxaemia *per se* on skeletal muscle. Rats were exposed for 4 weeks to chronic hypobaric hypoxia equivalent to a PO_2 of 410 mm Hg. As in other studies, a 10 % muscle atrophy was observed in the plantaris, an important muscle for locomotion, indicating that hypoxia could be an significant factor contributing to muscle atrophy in conditions where oxygen supply is compromised. It was found that the adaptation to chronic hypoxia was region- rather than fibre type-specific: capillary proliferation occurred only in the deep, oxidative region, while only in the superficial glycolytic region did mitochondrial biogenesis occur. The implication for patients with COPD is that some muscles or muscle regions may be more or less affected by hypoxia than others.

The aetiology of skeletal muscle dysfunction and its relationship to reduced exercise tolerance are poorly understood. The results from this thesis on smokers before they developed COPD and rats exposed to chronic hypoxia provide indications that smoking and hypoxia might contribute to peripheral fatigue and muscle atrophy in clinical situations. In the future, the relative contribution of smoking (amongst other factors, such as hypoxia, inflammation and disuse) should be studied to come to an integrative theory about muscle dysfunction and fatigue in patients with COPD.

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APPENDIX

METHODOLOGY

In this appendix, the procedures described in this thesis are explained in more detail. First, the *in vivo* measurements of skeletal muscle size and function are described including some comparison of data obtained on a custom-made isometric chair with those that were gathered from the same subjects on the apparatus used in this body of the work. Finally, the detailed protocols of the histochemical staining methods and analyses are provided and discussed.

In vivo measurements of skeletal muscle size and function

Anatomical cross-sectional area

The anatomical cross-sectional area (ACSA) of the quadriceps was measured with magnetic resonance imaging (MRI), using a fixed 0.2 T MRI scanner (E-Scan, ESAOTE Biomedica, Genova, Italy), at 50% of femur length. Using ultrasound, the head of the trochantor and the most distal point of the femur were identified with the participant in a supine position (with the knee joint in an extended position). Reference markers were placed along the upper leg on the skin to indicate 50% of the total length. Subjects were supine for 15 min prior to the scan to make sure any fluid shifts had stabilised.

After pilot studies, the following scanning parameters were chosen to give the best images of the four heads of the quadriceps muscle. Scans were obtained with a T1 weighted, high resolution, gradient echo profile, with the following scanning parameters: echo time: 0.016 s; repetition time: 0.1 s; field of view: 0.33 x 0.254 m and a slice thickness of 0.005 m.

Muscle function: Cybex dynamometer

The type of Cybex norm dynamometer (Ronkonkoma, New York, USA) used in these studies is widely used for measurement of *in vivo* skeletal muscle contractile properties (Harridge *et al.*, 1996; Reeves *et al.*, 2004; Morse *et al.*, 2007). All torque measurements were performed on the right quadriceps femoris muscle. Participants were seated with the hips at 90° flexion and shoulders strapped to minimise extraneous movements. The lateral epicondyle was aligned with the axis of rotation of the dynamometer identified by a laser beam from the centre of rotation of the dynamometer. Since the maximal voluntary torque requires the participants' maximal effort, they were first familiarized with the setup on a separate occasion and

during the actual data collection visual feedback of the torque signal was given together with verbal encouragement (McNair *et al.*, 1996). |Maximum voluntary contractions (2-3 seconds each) were repeated 2 or 3 times at knee joint angles of 60, 70 and 80˚ (full extension=0˚) in a random order with two minutes rest between each contraction to prevent the development of fatigue. The angle at which the highest torque was achieved was defined as the optimal joint angle and the maximum torque was the highest torque achieved during the three repetitions at that angle. All further measurements were done at optimal knee joint angle.

Percutaneous muscle stimulation

Percutaneous electrical stimulation (square wave, pulse width 50 μs; DSV Digitimer Stimulator, Digitimer Ltd., Herts, UK) was applied using carbon-rubber pads (76 mm x 127 mm, Versastim, Conmed Corp., N.Y., USA). The anode was placed over the proximal region of the quadriceps and the cathode over the distal third of the upper leg. The voltage was set at 400 V and was kept constant during the course of the experiment.

Voluntary activation

To assess the ability of the subjects to activate the knee extensors during isometric contractions, voluntary activation (VA) levels were determined using a variant of the interpolated twitch technique (Shield & Zhou, 2004). To increase the sensitivity of the test, a doublet (inter-pulse interval 10 ms) was applied instead of a single twitch. The current used to test for activation was determined using single pulses with the subject in a relaxed state, starting from 100 mA, and increasing until no further increase in torque was observed. The first doublet was applied with the subject in a relaxed state and a second (interpolated) doublet was delivered during the plateau phase of the MVC. The ratio of interpolated and resting doublets was used to provide an index of activation (Allen *et al.*, 1995) as follows:

$$\text{Voluntary activation (\%)} = 100 \times \left(1 - \left(\frac{\text{superimposed doublet torque}}{\text{resting doublet torque}}\right)\right)$$

where the superimposed doublet torque is the additional torque during the MVC caused by the doublet.

Torque-frequency relationship

The stimulation current for the determination of the torque-frequency relationship and fatigue protocols was adjusted so that a 1-s 100-Hz tetanus produced ~30 % of maximal voluntary isometric torque. Five minutes after the last MVC, the quadriceps muscle was stimulated with 5 single twitches at 1Hz and then 1s tetani at 10, 15, 20, 30, 50 and 100 Hz applied in random order, each separated by 1 min.

Fatigue tests

Five minutes after determining the torque-frequency relationship of the quadriceps muscle, the resistance to fatigue was determined by a series of electrically evoked isometric contractions (60 contractions, 30 Hz, 1 s on 1 s off (or 0.5 s off) at the current used to determine the torque-frequency relationships). Generally the test was carried out with the circulation to the leg intact but in one series the blood supply to the leg was occluded with a pneumatic thigh cuff (Accoson, Harlow, UK) inflated to ~240 mm Hg.

Analysis of variables

All torque signals were sampled at a frequency of 2000 Hz (Acknowledge, Biopac Systems, Santa Barbara, CA, USA) and off-line analysis was performed using Matlab (Matlab, the Mathwork Inc., S. Natik, MA, USA). Each torque signal was filtered with a low-pass fourth order Butterworth filter with a 30 Hz cut-off frequency to reduce the noise in the signal.

From the 30 or 100 Hz tetanus of the frequency-torque relationship, the contraction time was measured as the time from activation until 90% of the maximal torque was reached, and the relaxation time as the time for the torque to decline to 50% of maximal torque after cessation of stimulation. The maximal rate of relaxation (MRR, s^{-1}) was determined from the 30 Hz tetanus and calculated as the lowest value of the differentiated torque signal expressed relative to the highest torque recorded during that contraction.

The fatigue index was expressed as the highest torque recorded during the last contraction divided by that during the first contraction (expressed as a %). To obtain an indication of the time course of fatigue, the torque and maximal relaxation rate (MRR) for each contraction during the fatigue protocol were expressed as percentages of the initial value.

Compliance in the system

One of the key parameters for assessing contractility is the maximal rate of contraction and relaxation (MRC and MRR respectively). To ensure a rapid contraction and relaxation, the system needs to be rigid, such that the torque exerted by the muscle can be transferred to the transducer without first stretching a compliance. Two factors that affect the transduction of the torque exerted by the muscle to the transducer of the Cybex dynamometer are the compliance of the patella tendon and the stiffness of the Cybex dynamometer. The first is a biological factor and was not of principle interest in the present study. Therefore, no measurements have been made to account for this factor. Tendon compliance is related to the physical activity level of the participant (Reeves *et al.*, 2003; De Boer *et al.*, 2007), and the participants in the present studies were carefully matched for physical activity level. It is unlikely therefore that the results discussed in Chapter 2 (gender-related differences in fatigue resistance) and in Chapter 3 and 4 (smokers *vs.* non-smokers) are explicable by differences in the tendon compliance. Further studies, however, might be interesting to determine the contribution of tendon compliance to the torque-frequency relationship and fatigue resistance since the greater the compliance the more movement and work is done and this would be reflected in the energy demands of the fatigue tests.

The other factor, the stiffness of the Cybex dynamometer has been reduced as far as possible. The shin pad was modified such that the cushion was replaced with a more rigid pad and care was taken when strapping the participant in the seat (Figure 9.1A). In addition, a counterweight was placed on the Cybex lever arm to decrease the slack in the system. However, these changes have not totally ensured the measurements were 'slack-free', as it was observed in a previous study that the knee joint can move up to 20 degrees during a maximal voluntary contraction (MVC) (Tsaopoulos *et al.*, 2007). We have recently obtained a custom-made isometric chair (MMUscle) that is stiffer, and specially designed by the Department of Physical and Medical Technology at the VU University Amsterdam (Figure 9.1B), which allows testing the contractile properties of the quadriceps muscle with a stiffer system.

Figure 9.1. (**A**) Participant in the Cybex NORM dynamometer. The anatomical axis of rotation of the knee joint was aligned with the machine's axis of rotation and safety stops placed at the extremes of extension and flexion. Note the weights on the lever arm used to reduce the slack in the system. (**B**) Participant in the MMUscle chair. No alignment of the axis of rotation of the knee joint is needed, as the forces are transferred through the shin pad.

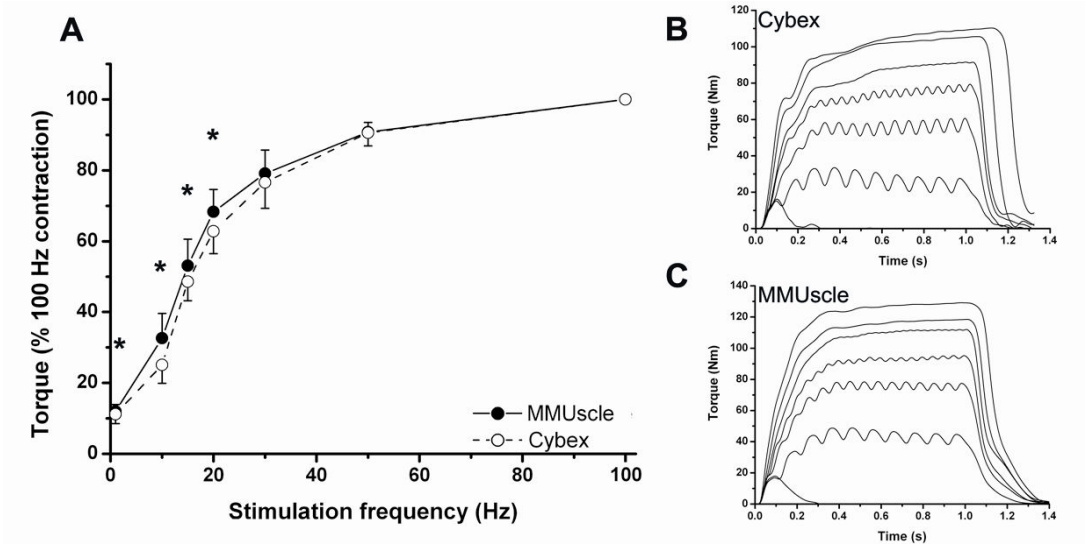

Figure 9.2. Torque-frequency relationship of four healthy men tested on the Cybex and on the MMUscle chair (**A**). A significant left-shift was found when tested in the MMUscle chair. In (**B**) and (**C**) a typical example of one participant on both systems are shown. Note the smoother rise in torque in the stiffer set-up (**C**). Values are shown as mean and SD. * denotes significant difference ($P<0.05$).

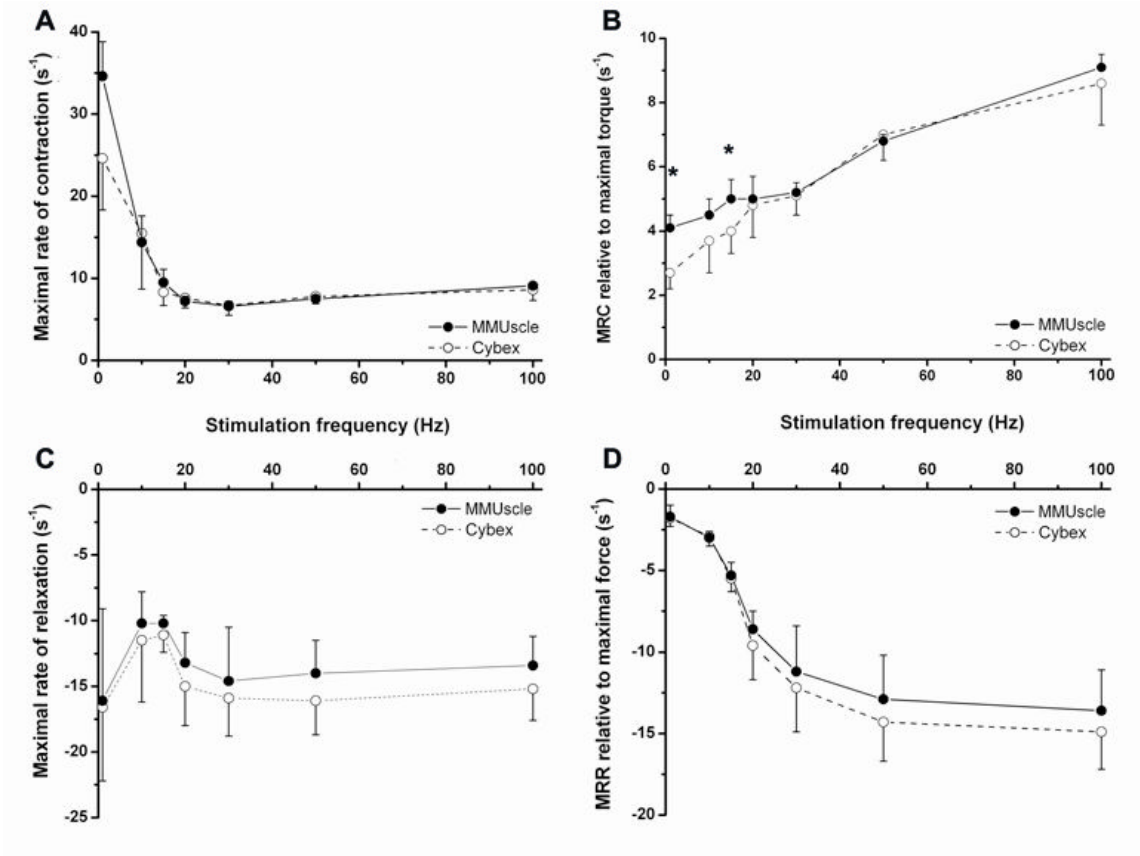

Figure 9.3. The maximal rate of contraction (MRC, **A** and **B**) and relaxation (MRR, **C** and **D**) were determined from each frequency during the torque-frequency relationship and calculated as the highest and lowest value of the differentiated torque signal respectively. In **A** and **C** this value was expressed relative to the highest torque recorded during the same contraction, while this value was expressed as the highest torque recorded during the 100 Hz contraction in **B** and **D**. This enables comparison between the stiffness of both systems. Significant differences at lower frequencies were observed in the MRC (**B**, $P<0.05$), but not in the MRR (**D**).

In a validation study the contractile properties of four healthy male volunteers were compared on both systems. Figure 9.1 shows a representative example of one participant on both systems.

The maximal rate of contraction (MRC) and relaxation (MRR) were determined at each frequency during the torque-frequency relationship and calculated as the highest (MRC) and lowest (MRR) value of the differentiated torque signal normalised to the maximal torque during that contraction. Because the higher contribution of the slack during the lower frequencies (especially the single twitch), the MRR and MRC were expressed relative to the highest torque recorded during the 100 Hz contraction. The torque-frequency relationship was found to be significantly shifted to the left in the stiffer MMUscle chair ($P<0.05$; Figure 9.2A). This left-shift was mainly caused by a significantly higher MRC relative to the maximal torque at 100 Hz at the lower

frequencies (Figure 9.3B), resulting in a more rapid transmission of torque from the muscle to the transducer.

The results from this validation study show that the torque-frequency relationship can be affected by the stiffness of the system on which the participant is tested. This is mainly due to a faster and therefore higher rise in torque at the lower frequencies (1-15 Hz). Paradoxically, the oscillation in the 10, 15 and 20 Hz torque signals seem to be slightly less when tested with the MMUscle chair. An explanation of this might be that the contractions in the Cybex are not fully isometric, so the muscle fibres themselves are shortening, resulting in a lower torque (due to the torque-velocity relationship) during contractions with a low stimulation frequency. This might also explain the significantly quicker rise in torque in the MMUscle chair at lower frequencies (Figure 9.3B). At higher frequencies, the results from the Cybex seem to be comparable with the MMUscle chair (Figure 9.3B and D).

The relationship between contractile properties and muscle fibre type

A factor that has received little attention in the literature is the extent to which the *in vivo* contractile properties of the quadriceps muscle are related to the fibre type distribution of the muscle. From the data shown in the Introduction (Fig 1.X) , rat muscles such as the soleus muscle have slower maximal rates of contraction (MRC) and relaxation (MRR) compared to fast muscles, such as the EDL muscle. However, in a mixed muscle, such as the quadriceps muscle, the relationship between fibre type and contractile speed is less obvious (Wiles *et al.*, 1979). In the few participants from which both the MRR and the fibre type of the quadriceps muscle (n=10) were obtained no correlation (R^2=0.47; P=0.163) was observed between MRR at 30 Hz and the area percentage of type I. Nevertheless, it was interesting to note that one old male participant in this study had only type I fibres in the quadriceps muscle (99.7 %), and also had the slowest MRR at 30 Hz (-11.1 s^{-1}), compared to -15.0 ± 2.3 s^{-1} in the others with 52.4 ± 11.0 % type I fibres. The cause of the absence of a strong relationship might relate to the distribution of fibre types within the quadriceps muscle (Lexell *et al.*, 1983). As was also observed in the rat plantaris muscle (Figure 7.3 and 9.4), a predominance of type I fibres has been shown in the deeper regions of the quadriceps muscle (Lexell *et al.*, 1983). Therefore, it may not be possible to extrapolate the fibre type composition obtained from one specific region of the vastus lateralis muscle to the whole quadriceps muscle group, and hence directly

relate fibre type composition to *in vivo* contractile speed. Nonetheless, a significant correlation (R^2=0.40, P=0.002) existed between the MRR and the results from the fatigue test under ischemia (Figure 2.4), indicating that the slower the muscle at the start of the fatigue test, the less the muscle will fatigue which is consistent with the expected behaviour of different mixtures of fibre types.

Histochemical staining methods

Results of histochemical staining methods on sections of muscle tissue of rats and humans are reported and discussed in Chapter 5, 6 and 7.

Wistar rats (11-week-old, all male) were kept at room temperature and a 12 h light-dark cycle. Food and water were given *ad libitum*. They were killed by an intraperitoneal injection of an overdose of pentobarbital sodium (70 $mg \cdot kg^{-1}$). The plantaris muscles was carefully excised, blotted dry, weighed, slightly stretched to minimise bias related to tortuousity of capillaries, directly frozen in liquid N_2 and stored at -80^{o}C. All protocols and procedures were approved by the University of Nijmegen Institutional Animal Care and Use Committee.

Eleven healthy participants volunteered to provide a biopsy of their vastus lasterlis muscle. Written informed consent was obtained from each participant prior to taking the percutaneous biopsy. All procedures were approved by the local ethics committee of the Manchester Metropolitan University (UK). The entire procedure was carried out under aseptic conditions. After local anesthesia with 2 % lidocaine, a small incision was made with a scalpel and the percutaneous biopsy of the vastus lateralis muscle (at ~50 % of the muscle length) was obtained using a conchotome. The wound was closed with steristrips and covered with bandage for up to 2 days. The biopsy was placed on cork with the longitudinal axis of the fibres perpendicular to the surface of the cork, quickly frozen in liquid N_2 with vigorous shaking to prevent development of ice-crystals, and stored at -80^{o}C.

Histochemistry

Transverse serial 10-µm sections were cut on a cryostat at -20°C and mounted on polylysine-coated slides. The sections were stored at -80°C until further analysis, unless otherwise stated. All sections were mounted in glycerine-gelatine after staining. The thickness of the slices was verified by cutting 10 µm sections of a gelatine block containing a homogeneous myoglobin concentration (see below) on a

recently calibrated cryostat and compared to identical sections cut on the cryostat where the human and rat samples were cut. Serial sections were used to identify the same fibres stained for different enzyme activities or structural features. In this way, fibre type, SDH-activity, capillary density and myoglobin concentration were determined in the same fibre. To our knowledge, this is the first study that has determined all this number of variables in the same muscle fibre.

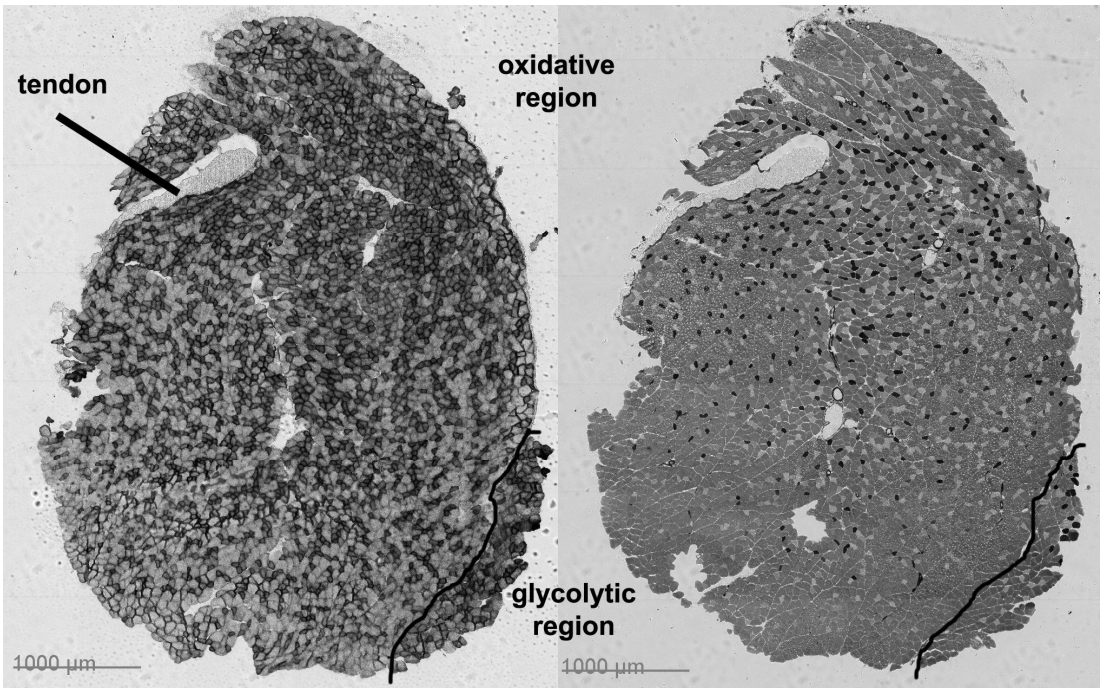

Figure 9.4. Example of a plantaris muscle of the Wistar rat (taken from chapter 7). On the left, the section is stained for SDH-activity (see text), while the right serial section was stained for acid myofibrillar ATPase (pH=4.55). The dark fibres on the right are classified as type I, while the white fibres are classified as type IIa and the grey fibres as type IIx/b. One can observe that the type I and IIa fibres have, as expected, a higher SDH-activity. Note that on the right hand corner of the muscle, a small part of the gastrocnemius muscle is still visible.

In the rat plantaris muscle two regions with different fibre type composition can be distinguished (Figure 9.4); near the tendon, a higher proportion of type I fibres (and SDH-activity) can be observed (this region is referred to as the deep, or oxidative region), while at the other side of the muscle hardly any oxidative fibres can be found (referred to as the superficial, or glycolytic region).

Determination of fibre type

Fibres were classified as type I, IIa, or IIx (IIx/b in rats) based on the pH sensitivity of their myofibrillar ATPase, using the methods first described by Brooke & Kaiser

(1970); type I myofibrillar ATPase is acid stable and alkaline labile, while type IIa myofibrillar ATPase is acid labile and alkaline stable with that of IIb/x in between. The fibres thus appear dark, white and grey after acid pre-incubation, respectively. Sections were pre-incubated for 10 minutes in a buffer containing 0.1 M NaAc and 0.1 M KCl at pH 4.55 set with acetic acid, quickly rinsed in 20 mM glycin and 20 mM $CaCl_2$ at pH 9.4 (set with NaOH) and subsequently incubated for 25-30 minutes at 37°C in 40 mM glycine, 20 mM $CaCl_2$ and 2.5 mM ATP at pH 9.4 (set with NaOH). Sections were quickly rinsed with 1% $CaCl_2$, left in 2% $CoCl_2$ for 3 minutes, washed with distilled water and coloured in 2% $(NH_4)_2S$ and washed in water.

Determination of SDH-activity

Succinate dehydrogenase (SDH) is an enzyme in the citric acid cycle and part of the respiratory chain. It has been shown by our lab that the calibrated histochemical method described below is linearly related to the maximum rate of oxygen uptake by the muscle fibre (Van der Laarse *et al.*, 1989). Immediately after cutting, sections were dried for 15 min and stained for SDH-activity (Pool *et al.*, 1979; Van der Laarse et al., 1989). Sections were incubated at 37°C in the dark for 20 min in a medium consisting of 37 mM sodium phosphate buffer (pH 7.6), 74 mM sodium succinate and 0.4 mM tetranitroblue tetrazolium (TNBT). The reaction was stopped with a 30 s incubation in 0.01 M HCl and fixed in 10% formalin for 20 min. The staining intensity was determined as the optical density of the final reaction product using an interference filter of 660 nm at a magnification of x 20 (ImageJ, National Institute of Health, Bethesda, USA). A 3rd order calibration line was obtained before scanning each section using grey filters with a known absorbance. By carefully outlining the muscle fibre, the mean optical density for each fibre was obtained. After subtracting the optical density of the background (a part of the section that did not express SDH-activity), optical density values were converted to staining rate, expressed as absorbance units (ΔA_{660}) per micrometer section thickness per second of incubation time (ΔA_{660} μm^{-1} s^{-1}). Besides giving an estimate of the aerobic capacity of the fibre, another advantage of this method is that values from different samples and studies can be compared, as the values are calibrated.

SDH-activity and physical activity level

The human participants discussed from Chapter 6 were matched for their physical activity level, which was assessed with a physical activity questionnaire (Baecke *et al.*, 1982). The questionnaire has been validated with pedometers (Baecke *et al.*, 1982) and with maximal oxygen uptake (Jacobs *et al.*, 1993; Richardson *et al.*, 1995). In addition, the data in the thesis show a strong correlation (r=0.83, P=0.001) between the overall SDH-activity (weighted for fibre type) and physical activity level (Figure 9.5).

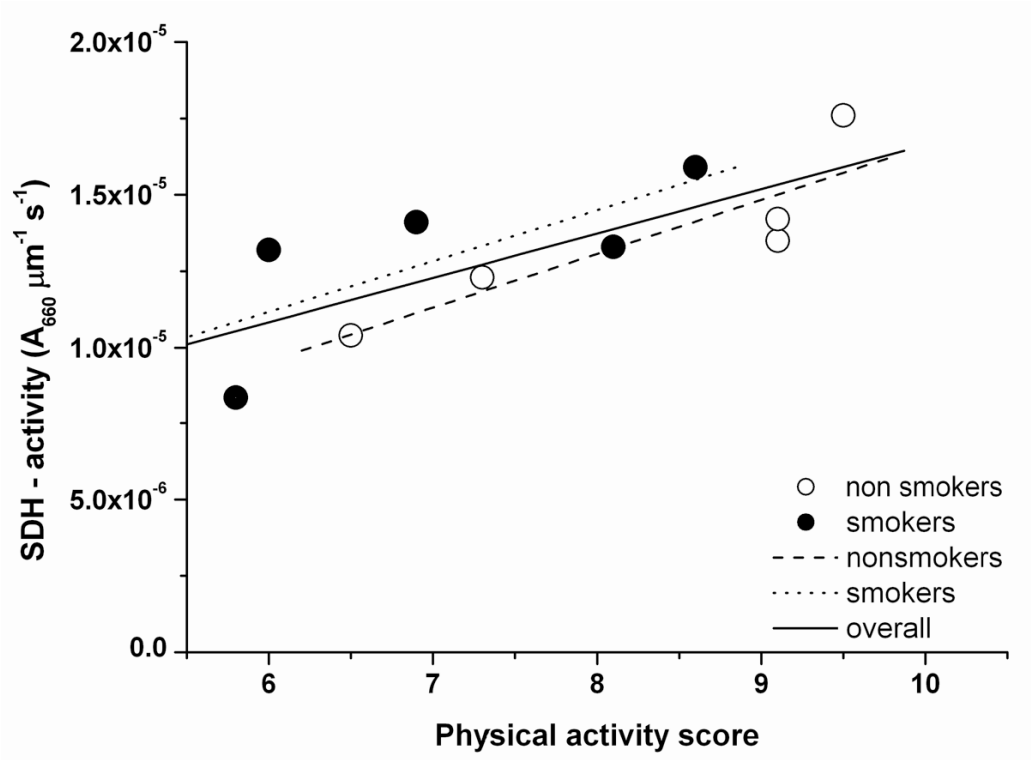

Figure 9.5. Relationship between physical activity score measured with the Baecke questionnaire and the oxidative capacity, measured as the SDH-activity. Pearson's correlation coefficient for non-smokers is r=0.87 (P=0.06) and r=0.88 (P=0.01) for smokers. Overall, the correlation coefficient was 0.83 (P=0.001).

Determination of capillarisation

Sections were stained with alkaline phosphatase (rat; Degens et al., 1992) or lectin (human; Ahmed et al., 1997) to identify capillaries.

Rat sections were fixed in chloroform-acetone (1:1; 4°C), quickly rinsed in distilled water and subsequently stained for 1 h in 0.01% nitro blue tetrazolium (NBT) and 0.002% 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt in 6.9 mmol $MgSO_4.7H_2O$ and 27.5 mmol $Na_2B_4O_7.10H_2O$ buffer, adjusted to pH 9.2 with boric

acid. After quickly rinsing the sections in distilled water and a post-fixation in 10% formalin (4% formaldehyde), slides were mounted in gelatine-glycerol.

Human sections were fixed in acetone (4°C), kept in HEPES for 15 minutes, incubated in PBS containing 0.9% 30% H_2O_2 for 30 minutes to reduce background staining and subsequently incubated with lectin (*Ulex Europeus*) in HEPES with 1% BSA (2000 µg/ml). The capillaries were visualised using a Vectastain ABC and DAB substrate kit (Vector Laboratories, Peterborough, UK) before mounting in gelatine-glycerol.

Capillaries were analyzed using the method of capillary domains (Hoofd *et al.*, 1985; Degens *et al.*, 1992). This method not only provides the overall indices of capillary to fibre ratio (C/F) and capillary density (CD), but also provides measures of the capillary supply to individual fibres of different fibre types, even when they lack direct capillary contacts. Since the deep and superficial region of the muscle differ in their fibre type composition and capillary supply, these regions were analysed separately. Photomicrographs of cross-sections of the human vastus lateralis muscle and the deep and superficial region of the rat plantaris muscle were taken. The fibre type distribution was assessed and the outlines of the muscle fibres as well as location of each capillary were digitised on a calibrated digitising tablet (Model MMII 1201, Summagraphics Digitisers, Austin, Tex, USA). The overall capillary density (CD) was defined as the number of capillaries mm^{-2} of tissue. Capillary domains, defined as the area surrounding a capillary delineated by equidistant boundaries from adjacent ones, were constructed and their surface area calculated. From the overlap of the domains with muscle fibres, the local capillary to fibre ratio (LCFR) and the capillary fibre density (CFD) were obtained. The LCFR for a fibre was defined as the sum of the fractions of each domain area overlapping the fibre. This variable has a continuous distribution and is more sensitive than e.g. capillaries around a fibre. It also allows determination of the capillary supply to a fibre even when it lacks direct capillary contacts. Such situations were observed occasionally in the glycolytic region of the rat plantaris muscle. CFD, which is LCFR divided by the FCSA, provides the capillary density of that fibre and is expressed as the number of capillaries mm^{-2}.

Determination of myoglobin concentration

The myoglobin concentration in individual muscle cells was determined as described previously (Van Beek-Harmsen *et al.*, 2004). Frozen sections were defrosted in vacuum to avoid condensation and consequent redistribution of myoglobin. Sections were then vapour-fixed for 1 h in paraformaldehyde at 70°C. Subsequently, the sections were fixed for 10 min at room temperature in 2.5% glutaraldehyde in 0.07 M sodium phosphate buffer (pH=7.4) and quickly rinsed with distilled water. To detect myoglobin sections were incubated for 1 h at room temperature in a medium containing 59 ml 50 mM TRIS/80 mM KCl buffer (pH=8.0), 25 mg ortho-toluidine dissolved in 2 ml 96% ethanol (at ~50°C) and 1.43 ml 70% tertiary-butyl-hydroperoxide. Sections were then washed with distilled water, mounted and scanned on a calibrated DMRB microscope (Leica, Wetzlar, Germany) with an interference filter of 436 nm. A 3rd order calibration line was obtained before scanning each section using grey filters with a known absorbance.

The red blood cells in the capillaries contain haemoglobin which was visible as dark spots at the edges of the muscle fibres. The myoglobin concentration was, however, homogeneously distributed within the cell and therefore the mean optical density was obtained from the centre of each fibre. After subtracting the optical density of the background (a part of the section that did not contain myoglobin), optical density values were converted to absorbance values. Using a calibration line, obtained from absorbance values from 10 μm sections cut from gelatine blocks containing 0.1, 0.2, 0.3 and 0.4 mM myoglobin, absorbance values were converted into concentrations (Van Beek-Harmsen *et al.*, 2004).

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